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SOME BIOCHEMICAL STUDIES ON THE PRODUCTION
OF ETHYLENE BY A PARTICULATE FRACTION FROM RIPENING TOMATOES

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

by

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EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Some Biochemical Studies on the Production of Ethylene by a Particulate Fraction from Ripening Tomatoes", submitted by Michael Meheriuk, B.Sc., B.Ed., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Date *August 20, 1965*.....

ABSTRACT

Studies were made with a particulate fraction from tomatoes to assist in the elucidation of the pathway for the biosynthesis of ethylene.

Although several substrates were evaluated, only a few were effective in promotion of ethylene production when they were added to the particulate fraction. These included ethanol, propionic acid, serine, aspartic acid, glutamic acid, β -alanine and γ -aminobutyric acid. Stimulation of ethylene production was achieved only with thiamine pyrophosphate and magnesium ions, α -lipoic acid and nicotinamide adenine dinucleotide phosphate, of the co-factors tested. All of the cations studied were found to be either without effect on, or inhibitory to, ethylene evolution. A rather broad pH range was observed for ethylene production in the system, with severe inhibition occurring at pH values of 3.0 and 8.0.

Studies with inhibitors revealed roles for thiol groups and disulfide linkages in ethylene biosynthesis. Carbonyl groups were demonstrated to be essential in the process. Metalloproteins or cations were shown to be involved in the synthesis of the volatile and the importance of the cytochrome system to ethylene biogenesis was suggested. Transaminase activity was implicated also.

Attempts to construct an enzyme "complex" metabolizing β -alanine to ethylene were successful to a degree and combinations of factors found to stimulate ethylene production from

β -alanine were thiamine pyrophosphate, magnesium ions, α -lipoic acid and flavin adenine dinucleotide or flavin mononucleotide, and thiamine pyrophosphate, magnesium ions and nicotinamide adenine dinucleotide phosphate.

The contribution to the total yield of ethylene by dehydration of ethanol in mercuric perchlorate and from the process of sonication were shown to be of minor importance.

A tentative proposal was made for the pathways of biosynthesis of ethylene on the basis of evidence from the experiments described above. Unequivocal proof of the hypotheses awaits further experiments but however incomplete this study may be, it should, nevertheless, provide useful information for those working with purified enzyme preparations.

Acknowledgments

I should like to take this opportunity to express my humble "merci beaucoup" to Dr. Mary Spencer for her guidance, suggestions and criticism throughout the entire course of this research. It is, indeed, rewarding to have as a supervisor a person who takes deep interest in the work and for whom the individual is of sincere concern.

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TABLE OF CONTENTS

<u>Chapter</u>	<u>Page</u>
1. Introduction	1
2. Methods	10
1. Preparative Procedures	10
a) Selection and Storage of Tomatoes	10
b) Grinding	11
c) Centrifugation	11
2. Ethylene Collection Methods	12
a) Suspension and Sonication of Particles	12
b) Collection Apparatus	13
c) Conditions during Collection	13
d) Collection Periods	14
3. Analysis of Ethylene	14
a) Preparation	14
b) Liberation of Samples and Preparation of Standards	14
c) Injection of Samples	16
d) Sensitivities Employed and Limit of Analysis	16
e) Calculation of Ethylene Content	17
f) Nitrogen Determinations	17
g) Chromatographic Unit	18
4. Procedures Used in Various Studies	19
a) Study of Cofactors, Substrates and Inhibitors	19
b) pH Studies	20
c) Ageing Studies	20
5. Additional Experimental Controls	21
a) Sonication Controls	21
b) Ethanol Controls	21
6. Confirmation of the Identity of Ethylene	24
a) Absorption by Bromine Water	24
b) Mass Spectrometry	24
3. Effects of Various Substrates on the Production of Ethylene	27
Results and Discussion	31
a) Glycolaldehyde	31
b) Glucose	32
c) Galactose	34

d)	Xylose	34
e)	Ethanol	35
f)	Propionic Acid	36
g)	Pyruvate	37
h)	α -Ketoglutaric Acid	38
i)	Aspartic Acid	39
j)	Glutamic Acid	40
k)	γ -Aminobutyric Acid	42
l)	β -Alanine	42
m)	Serine	44
n)	Glycine	44
o)	Glyoxylate	45
p)	Acrylic Acid	45
4.	Effect of Several Cofactors on Ethylene Production	54
	Results and Discussion	56
a)	Bovine Serum Albumin	56
b)	Coenzyme A	56
c)	Flavin Adenine Dinucleotide	57
d)	Flavin Mononucleotide	57
e)	Glutathione	58
f)	α -Lipoic Acid	59
g)	Nicotinamide Adenine Dinucleotide	59
h)	Nicotinamide Adenine Dinucleotide Phosphate	60
i)	Nicotinamide Adenine Dinucleotide and Coenzyme A	60
j)	Nicotinamide Adenine Dinucleotide Phosphate and Coenzyme A	61
k)	Pyridoxal	62
l)	Pyridoxal Phosphate	62
m)	Thiamine Pyrophosphate	63
5.	Effects of Various Inhibitors on Ethylene Produc- tion	70
	Results and Discussion	72
a)	Arsenite	72
b)	Aminooxyacetic Acid	74
c)	Azide	75
d)	p-Chloromercuribenzoic Acid	75
e)	Cyanide	76
f)	Cycloserine	78
g)	Diethyldithiocarbamate	79

h)	Fluoride	80
i)	Hydroxylamine	82
j)	Iodoacetamide	82
k)	Malonic Acid	83
l)	Mercuric Sulfate	84
m)	Monofluoroacetate	85
n)	Oxythiamine	86
o)	Semicarbazide	87
p)	Silver Nitrate	88
q)	L-Thyroxine	89
6.	The Effects of Several Cations on Ethylene Pro- duction	99
	Results and Discussion	102
a)	Mg ⁺⁺	102
b)	Mn ⁺⁺	103
c)	Fe ⁺⁺	103
d)	Cu ⁺⁺	104
e)	Zn ⁺⁺	104
f)	Co ⁺⁺	105
g)	Al ⁺⁺⁺	105
7.	Effects of pH on Ethylene Production	109
	Results and Discussion	110
a)	pH 3.0	110
b)	pH 5.0	110
c)	pH 6.0	111
d)	pH 8.0	111
e)	pH 9.0	111
8.	Effects of Cofactors and Substrates with β -alanine on Ethylene Production	115
	Results and Discussion	116
a)	Coenzyme A	116
b)	Flavin Adenine Dinucleotide	117
c)	Flavin Mononucleotide	118
d)	α -Ketoglutaric Acid	118
e)	α -Lipoic Acid	118
f)	Nicotinamide Adenine Dinucleotide	119
g)	Nicotinamide Adenine Dinucleotide Phosphate	
h)	Oxalacetic Acid	120
i)	Oxythiamine	120

<u>Chapter</u>	<u>Page</u>
j) Pyridoxal Phosphate	121
k) Thiamine Pyrophosphate	121
9. Effects of Cofactors and Substrates with β -alanine Decarboxylation System I on Ethylene Production ..	127
Results and Discussion	128
a) Coenzyme A	128
b) Flavin Adenine Dinucleotide	128
c) Flavin Mononucleotide	129
d) α -Ketoglutaric Acid	129
e) α -Lipoic Acid	130
f) Nicotinamide Adenine Dinucleotide	130
g) Nicotinamide Adenine Dinucleotide Phos- phate	131
h) Oxalacetic Acid	131
i) Oxythiamine	132
j) Pyridoxal Phosphate	133
10. Effects of Cofactors and Substrates with β -Alanine Decarboxylation System II on Ethylene Production .	138
Results and Discussion	139
a) Coenzyme A	139
b) Flavin Adenine Dinucleotide	140
c) Flavin Mononucleotide	141
d) α -Ketoglutaric Acid	142
e) Nicotinamide Adenine Dinucleotide	142
f) Nicotinamide Adenine Dinucleotide Phosphate	143
g) Oxalacetic Acid	144
h) Oxythiamine	144
i) Pyridoxal Phosphate	144
11. The Effects of Ageing on Ethylene Production in the Presence of Some Added Factors	149
Results and Discussion	150
a) Adenosine Triphosphate	150
b) Thiamine Pyrophosphate and Mg^{++}	150
c) Thiamine Pyrophosphate, Mg^{++} and β -alanine	151
12. The Identification, Estimation, and Possible Con- version to Ethylene, of Ethanol Produced by the Tomato Particulate Fraction	155
a) Identification	155
b) Estimation	155
c) Possible Conversion to Ethylene	157

<u>Chapter</u>		<u>Page</u>
	d) Dehydration Studies with Ethanol	158
13.	The Effect of Sonication of Several Cofactors and Substrates	162
	Results and Discussion	162
14.	Confirmation of the Identity of Ethylene	165
	a) Absorption by Bromine Water	165
	b) Mass Spectrometry	165
15.	General Conclusions	168
16.	References	174

List of Tables

<u>Table Number</u>		<u>Page</u>
I	Effects of Several Substrates on Ethylene Production by a Particulate Fraction from Tomatoes ,	50
II	Effects of Several Cofactors on Ethylene Production by a Particulate Fraction from Tomatoes . . .	65
III	Effects of Various Inhibitors on Ethylene Production by a Particulate Fraction from Tomatoes . . .	91
IV	Effects of Several Cations on Ethylene Production by a Particulate Fraction from Tomatoes	107
V	The Effects of pH on Ethylene Production by a Particulate Fraction from Tomatoes	113
VI	Effects of Several Cofactors and Substrates with β -alanine on Ethylene Production by a Particulate Fraction from Tomatoes	123
VII	Effects of Several Cofactors and Substrates with β -alanine Decarboxylation System I on Ethylene Production by a Particulate Fraction from Tomatoes	135
VIII	Effects of Several Cofactors and Substrates with β -alanine Decarboxylation System II on Ethylene Production by a Particulate Fraction from Tomatoes	146
IX	The Effect of Ageing in the Presence of Several Constituents on Ethylene Production by a Particulate Fraction from Tomatoes	154
X	The Ethanol Content of a Particulate Fraction from Tomatoes	159
XI	Ethanol Content of Volatiles Evolved by a Particulate Fraction from Tomatoes	160
XII	Ethylene Production by Sonication of Several Constituents ,	164

List of Figures

<u>Figure Number</u>		<u>Page</u>
1	Mass Spectrogram of the Volatiles Produced by a Particulate Fraction from Tomatoes	161
2	Mass Spectrogram of a Standard Sample of Ethyl- ene	166
3	Mass Spectrogram of an Unknown Sample of the Collected Volatile	167

LIST OF ABBREVIATIONS

1. ATP : Adenosine Triphosphate
2. p-CMB : p-Chloromercuribenzoic acid
3. CoA : Coenzyme A
4. DIECA : Diethyldithiocarbamic acid
5. EDTA : Ethylenediaminetetracetic acid
6. FAD : Flavin Adenine Dinucleotide
7. FMN : Flavin Mononucleotide
8. GSH : Glutathione (reduced)
9. HMP pathway : Hexosemonophosphate Pathway
10. NAD : Nicotinamide Adenine Dinucleotide
11. NADP : Nicotinamide Adenine Dinucleotide Phosphate
12. TCA cycle : The Citric Acid Cycle
13. TCA : Trichloroacetic acid
14. TPP : Thiamine pyrophosphate
15. Tris : Tris-hydroxyaminomethane

Ethylene, an olefinic hydrocarbon produced by plants, continues to be extensively studied by plant physiologists. The reason for the attention being focused on this study lies in the importance the volatile commands in fruit ripening. When it was confirmed that ethylene initiated or hastened the ripening process in fruits, keen interest was expressed as to what biochemical changes were induced by the gas. A recent review (1) by Burg of the subject of ethylene includes consideration of the production of the olefin, the physiological responses it causes and the research concerning its biosynthesis.

One need only glance at a partial list of physiological responses attributed to ethylene, to realize what a maverick ethylene really is:

- a) stimulation of fruit ripening (2,3,4,5,6)
- b) hastening of chlorophyll degradation (2,7,8,9,10,11,12,13,14)
- c) inducement of epinastic response in seedlings (10,15,16,17,18)
- d) production of drowsiness in flowers (19)
- e) prevention of elongation of stems (10,15)
- f) initiation of growth of adventitious roots (10,20)
- g) blanching of celery (9,21)
- h) production of abscission of leaves (15,17,22)
- i) inducement of swelling of roots (23)
- j) softening of shucks in pecan harvesting (24)
- k) stimulation of isocoumarin production in carrots (25)

Although many interesting facts about ethylene have been uncovered they are meager insofar as understanding the biochemical function of the volatile is concerned. Hansen (26), Burg and Burg (27), and Lyons, McGlasson and Pratt (28) found that certain minimal concentrations of ethylene must reside within the fruit before ripening could proceed. From McGlasson and Pratt's (29) work it became apparent that a particular physiological state was prerequisite before ethylene initiated the onset of the climacteric period in cantaloupe fruit.

Ethylene production has been studied under different physical conditions such as temperature and gaseous environment to see if production was affected or not. When anaerobic conditions were employed in vivo, production of the gas decreased sharply or ceased (5,30,31,32). Pertinent to anaerobiosis was Burg and Thimann's (30) observation that apple plugs kept in an anaerobic atmosphere accumulated a precursor that was rapidly converted to ethylene when exposed to oxygen. A similar result, with subcellular particles from tomatoes, was reported by Meheriuk and Spencer (33) and, furthermore, it was found that ATP could substitute for the readmission of oxygen. The suggestion put forth by them was that reinstatement of aerobic conditions resulted in synthesis of high energy compounds, which subsequently were utilized in the production of ethylene.

Characteristic temperature effects may serve as an indication for the presence of enzymic activity in biological materials and in this respect, it was noticed that temperature altered ethylene evolution. Studies by several workers (5,30,

31,32,34,35,36,37,38,39,40,41) demonstrated that at low temperatures (i.e. 5°C) ethylene production declined sharply and at temperatures exceeding 40°C production usually stopped within a short time. The temperature range for maximum production was from 20°C to 30°C. Since enzymes from plant tissue generally function optimally within this range one could assume that synthesis of ethylene was enzyme dependent.

Papers by Morgan and Hall (42,43), Hansen (44), and Abeles and Rubenstein (45) indicated that ethylene production could be stimulated by the plant auxins; 2-4-dichlorophenoxyacetic acid, indoleacetic acid and naphthaleneacetic acid. Moreover, Morgan and Hall stipulated a relationship between ethylene production and auxin level. However, acceptance of such a relationship cannot be expressed until it can be clearly shown that auxins directly influence ethylene production as opposed to an indirect effect by virtue of their manifest physiological effect on tissue. Only one paper has appeared on elevation of auxin level by ethylene treatment, that by Michner (46) in which β -indolylacetic acid was increased.

The physiological effects mentioned at the beginning of the introduction dealt with actions of ethylene on whole tissue. How these effects manifest themselves at the cellular level is not known but Lyons and Pratt (47) recently reported that plant mitochondria swell in the presence of ethylene. Whether the swelling is an in situ effect or an anomalous one will be decided by further experimental work.

Research into the biosynthesis of ethylene has centered mainly about the administration of substrates, labelled and

unlabelled, to ethylene producing systems. The common citrus fruit mold, Penicillium digitatum, which can grow on a minimal medium (48), is extensively used for studies involving substrates. The advantage in using the fungus stems from its ability to grow on a one carbon source and consequently, carbon for the bio-synthesis of ethylene would be limited to this source, with the possible exception of that from carbon dioxide fixation. Some of the substrates found to either sustain or stimulate ethylene production in Penicillium digitatum and the investigators who used them are as follows:

- i) Fergus (49) - citric acid cycle intermediates
- ii) Hall (50) - fructose, galactose
- iii) Phan Chan Ton - glycerol, alanine, glucose
- iv) Spalding (51) - glucose, citric acid
- v) Gibson (52) - citrate, pyruvate, malate

Insofar as labelled substrates supplied to the mold are concerned, Phan Chan Ton (40) detected low activity in ethylene from glucose-U-C¹⁴. Wang and his group (53, 54) collected labelled ethylene when TCA (the citric acid) cycle intermediates or aspartate or glutamate were supplied and concluded that the TCA cycle was directly involved. Contrary to the results of Wang and his group were those of Gibson (52) who found that little of the Carbon 14 from succinate and fumarate appeared in ethylene. She also found ethanol and pyruvate to be relatively good in that respect. It is evident that the results derived from Penicillium digitatum are not only conflicting but inconclusive. Incidentally, the author has found from his experimental work

with the fungus that results tended to be inconsistent and duplication of any one result was difficult to achieve.

It may be argued that the mold is not suitable for tracer studies but results obtained from other tissues have been for the most part inconclusive also. Burg (55, 56) and Burg and Thimann (30,57) applied sucrose, glucose, acetate, glycerol, tyrosine and xylose, labelled specifically or uniformly, to apple plugs. Only acetate and glucose gave labelled ethylene, and these but weakly. More recently, Burg and Burg (58) disclosed that pyruvate, triose, and fumarate labelled ethylene moderately well.

Attempts have been made to study metabolism of labelled ethylene supplied to plant tissue. Buhler, Hansen, and Wang (59) recovered the label in fumarate and succinate. In Jansen's experiments (60,61), the label appeared mainly in aromatic compounds. The degree of incorporation measured by either laboratory was well below 1% of the total activity. An earlier attempt by Nelson (60) disclosed that less than 75% of the ethylene administered to ripening tomatoes could be recovered again. He concluded that the non-recoverable quantity was metabolized by the fruit. However, his method of detecting ethylene was considerably less sensitive than those used today and may have contributed to the rather high percentage of "metabolized ethylene" by its inability to detect all of the ethylene present.

Determination of an unknown metabolic pathway in whole tissue is both tedious and difficult and when an appropriate subcellular system can be isolated is the task simplified.

Preparation of a subcellular fraction capable of producing ethylene was prompted by Burg and Thimann's (63) postulation that an osmotic sensitive particle was responsible for ethylene production in apple tissue. A subcellular fraction active in ethylene production was isolated from ripening tomatoes by Spencer (64). Chandra and Spencer (65,66) prepared subcellular fractions from rat liver, rat intestinal mucosa, and Penicillium digitatum that were able to evolve ethylene, but disruption of the isolated particles by ageing and other physical means was necessary for a high production of the volatile. Particles prepared from apples and tomatoes by Norris, Craft and Lieberman (67) proved to be inactive. Burg and Burg (68) also failed to isolate an active fraction from apples. Small amounts of ethylene were obtained by Meigh (69) from a tomato particulate fraction when the isolation procedures of Chandra and Spencer were used. Mitochondria prepared by Gibson (70) from beef heart were also reported to produce the volatile. Lieberman and co-workers observed some activity from apple cytoplasmic particles with thiomalic acid as a substrate (71) but later a more active fraction was isolated that utilized linolenic acid (72). The fact that ethylene is evolved by the subcellular fractions just mentioned suggests that these cellular particles may be a primary locus for the synthesis of ethylene. Although considerable knowledge will be gained about ethylene biosynthesis from the use of subcellular systems, a complete elucidation of the pathway will probably not emerge until studies on purified enzyme powders able to catalyze the

synthesis of ethylene have been conducted. Active enzyme powders have already been reported by Abeles and Rubenstein (73) and Thompson and Spencer (74).

Respiratory inhibitors serve as a useful tool in the hands of a biochemist whenever enzyme systems are being studied. The study of inhibitors and their effects on ethylene production has by no means been exhausted. On the contrary, information is somewhat limited, although valuable results have been reported by several workers. Hansen (32) reported inhibition of ethylene production by high concentrations of cyanide and sulfide, but low concentrations had no effect. Dinitrophenol, an uncoupler of oxidative phosphorylation, inhibited production of the olefin by whole tissue (63,75). Lieberman and co-workers (76,77) suggested from the inhibition caused by DIECA and EDTA that a copper metalloprotein may be active in ethylene biosynthesis. Burg and Thimann (63) noted that inhibition caused by fluoride was reversed by the addition of ATP, but, the inhibitory effect of iodoacetamide could not be reversed by the latter. Gibson's (52) work with Penicillium digitatum included the use of cyanide, azide, iodoacetate, malonate and monofluoroacetate, and only the first three caused a significant decrease in ethylene production. A study of the effects of several inhibitors on a subcellular fraction from tomatoes by the author has been reported previously (33).

Non-enzymic synthesis of ethylene has received considerable attention lately, especially from the point of view that it may be involved in fruit ripening. Free radical formation of ethylene was first suggested by Meigh (69).

The basis for his statement was the appearance of ethylene from a boiled tomato particulate fraction and also from a solution of sucrose and ATP, both having been sonically treated. A model system from the non-enzymic production of ethylene was evolved by Lieberman and Mapson (76,78). In their system activated linolenic acid in the presence of cupric ions gave rise to ethylene. The author has also observed non-enzymic synthesis of the volatile but the reader's attention is directed to Chapter 13 of the dissertation for a discussion of the results.

Highly relevant to the topic of non-enzymic production of ethylene is the work on irradiation of fruit by Maxie and co-workers (79,80). Ripening fruit when subjected to low doses of γ -rays had an enhanced ethylene activity but fruit given a high dosage suffered irreversible damage. Young (81) noticed that only preclimacteric avocados were susceptible to an increased evolution of ethylene upon γ -irradiation, and fruit in the climacteric stage was unaffected. Irradiation probably generates free radicals that form ethylene but it is interesting to speculate whether disintegration of subcellular particles is a concomitant event. If so, the observation of Chandra and Spencer (65) that disruption of particles results in high ethylene production would be applicable to irradiation studies.

To what purpose or end does the entire study of ethylene metabolism lead plant physiologists? Foremost in immediate practical application is the possibility of de-

laying the ripening of fruits and thus keeping them in storage until required, or alternatively, ripening them when needed. There is, also, the knowledge to be gained about the biochemical changes that ethylene induces in plant tissue. It will become known whether the responses mentioned earlier are a manifestation of one major biochemical event or whether each stems from an individual biochemical event. An answer as to why damaged and infected tissue (31,82,83,84,85,86,87) respire large amounts of ethylene will be available. One will also understand why animals when subjected to ethylene should exhale it for a long period thereafter (88). Since ethylene production and ageing in plants are thought to be synchronous processes (89,90) it may not be long before it is known whether the general process of ageing is, in any way, associated with ethylene production. We may also learn if ethylene synthesis is a highly specialized trait in plants and only vestigial in some animals or whether it is a physiological process common throughout the plant and animal kingdoms. Whoever discovers the keys that unlock the doors to the understanding of ethylene metabolism will have ended a period of investigation lasting for more than half a century.

METHODS

1. Preparative Procedures:

a) Selection and Storage of Tomatoes:

Since ethylene production is at a maximum during the climacteric phase of fruits (32,37,91,92,93,94,95), tomatoes in this stage of development were selected for the experimental work. Tomatoes in the climacteric period were those with a bright orange to light red coloring and were firm in texture. Ripe tomatoes were not used because fewer particles could be isolated and also, the particles were found to be considerably less active in ethylene production than particles isolated from tomatoes in the climacteric period. Dickinson and Hanson (96) noted that mitochondria from post climacteric or ripe tomatoes were fewer in number and were highly sensitive to pH changes. The severe and largely unknown physiological changes occurring in mitochondria from senescent fruit would complicate a research program on ethylene production by subcellular particles.

Tomatoes of the V121 variety were grown in the university greenhouses and when their supply expired commercial Mexican or Californian tomatoes were purchased from a local fruit packer. The tomatoes were washed in distilled water, dried and packaged in 1000 gm lots. The packages were then placed in a freezer and stored at a temperature less than -15°C . Fruit was utilized before three weeks of storage because prolonged storage resulted

in loss of ethylene production activity of the particles.

Fresh tomatoes were not routinely used because a continuous supply could not be assured and consequently, considerable delay would have been encountered during the course of research. Earlier work by Chandra (97) showed that particles from fresh and frozen tomatoes behaved similarly in ethylene production. A disadvantage observed with unfrozen tomatoes was the high quantity of pectin isolated with the particles. The pectin was undesirable because it caused the thickening of the reaction mixture containing the particles.

b) Grinding:

The frozen tomatoes were sectioned by chisel and hammer and ground by means of a Waring Blendor in ice cold buffer (0.5 M sucrose, 0.5 M KH_2PO_4 and adjusted to pH 8.0 with NaOH). The proportions used were 1000 gm of fruit to 1000 ml grind buffer. A powerstat setting of 100 for 30 seconds followed by a setting of 50 for 30 seconds reduced the sectioned fruit to a homogenate of pH 6.8 to 7.5. The homogenate was then strained through four layers of cheesecloth to yield a filtrate ready for centrifugation.

c) Centrifugation:

Initial centrifugation at 4000 g for 5 minutes in a Servall model RC-2 ultracentrifuge fitted with a GSA rotor removed unfragmented cells, nuclei and chloroplasts. The supernatant was strained through broadcloth placed in a Buchner funnel under a low vacuum and any suspended or floating material was retained. The filtrate was then

centrifuged at 35,000 g for 15 minutes in a Spinco model L-2 ultracentrifuge equipped with a number 19 rotor. The pellet obtained from the latter centrifugation constituted the subcellular fraction used in the experimental work. This particulate fraction was previously shown by Chandra (65, 92) to be quite active in the production of ethylene.

2. Ethylene Collection Procedures:

a) Suspension and Sonication of Particles:

The pellet obtained from the second centrifugation was suspended in cold sucrose phosphate buffer (0.5 M sucrose, 0.125 M KH_2PO_4 , adjusted to pH 7.2 with NaOH) and dispersed by means of magnetic stirrer and bar. During dispersion the solution was kept in an ice bath to maintain a temperature close to 0° C. The suspension was then sonicated for 4 minutes at 1.2 amperes in a refrigerated (4° C) Raytheon DF-101 10 kc sonic oscillator.

Particles isolated from 1000 gms of fruit were usually suspended in a total volume of 75 mls of buffer. A lower yield of particles from some varieties of commercial tomatoes necessitated a reduction in total volume to 50 ml of buffer. Of the total volume a sufficient quantity of buffer was retained to rinse out the centrifugation bottles and suspension vessel and thus, quantitative transfer of particles to the reaction flasks was insured.

For the ageing experiments of Chapter 11 of the dissertation, the particles were placed into the reaction vessel and no initial sonication of the suspension was

performed.

b) Collection Apparatus:

The reaction flasks were of the extraction type, with a capacity of 50 ml and prior to sonication of the particles calculated quantities of substrates, cofactors or inhibitors were weighed into them. Immediately after sonication, the suspension was gently stirred with a glass rod and then 25 ml of it was pipetted into a reaction flask. The apparatus and procedures used in the collection of the volatile were those of Chandra and Spencer (97,98).

c) Conditions during Collection:

The particulate suspension in each flask was stirred at low speed by a small stirring bar activated by a magnetic stirrer placed 2 inches below the flask. A piece of insulating material such as foamed styrene was placed on the stirrer to minimize heat transfer to the reaction vessel and its contents.

Room temperature was employed for all the experimental work except where specifically stated.

Maintenance of a pH value near 7.2 presented no difficulty because of the high molarity of the buffering agent (0.125 M KH_2PO_4). However, highly acidic organic and amino acids would have lowered the pH of the suspensions to 6.5 and less. In order to overcome the latter difficulty concentrated solutions of these acids were prepared and adjusted to a pH of 7.2 with NaOH. A specified quantity was then pipetted into the flask to give the desired molarity. Acids that required the treatment

described include glutamic, aspartic and α -ketoglutaric.

d) Collection Periods:

Ethylene production from the particles was studied over a period of 24 hours. The collection periods were 0-1 hr., 1-3 hr., 3-22 hr., 22-23 hr. and 23-24 hr. or consecutive times of 1, 2, 19, 1 and 1 hours, respectively. At the end of the 3-22 hr. collection period (or overnight ageing) the particulate suspensions were sonically treated again for 4 minutes at 1.2 amperes. Suspensions used in the ageing studies were given a similar sonic treatment at the end of this collection period. Clean, dry collection tubes (98) were used for every collection period to avoid contamination of samples by previous ones.

3. Analysis of Ethylene:

All of the ethylene samples collected in the experimental work were analyzed by gas chromatography.

a) Preparation of Samples:

Serum bottles containing 4.0 ml of the original 4.5 ml of mercuric perchlorate solution were tightly stoppered with serum stoppers and stored in a refrigerator at 5°C. Storage beyond one week was avoided because the ethylene complex formed is known to deteriorate with ageing (99).

b) Liberation of Samples and Preparation of Standards:

The ethylene was released from its mercuric complex

by the addition of 1 ml of 4 N LiCl to each serum bottle. The bottles were then immediately resealed and shaken for 20 minutes, during which time complete liberation of the ethylene from the complex took place (100).

The ethylene standards were prepared by pipetting into a duplicate series of serum bottles 1, 2, 3 and 4 ml of a calibrated stock solution containing from 0.15 to 0.20 $\mu\text{l/ml}$ of ethylene. Each serum bottle was made to a volume of 4.0 ml with the perchlorate reagent. Liberation of ethylene in the standards was accomplished by the same procedure as for the samples and was done at the same time as the samples. The fourth standard in the series (0.60 to 0.80 $\mu\text{l C}_2\text{H}_4$) was sufficient to give full scale deflection on the recorder at a sensitivity of 50 mV.

Blanks containing only mercuric perchlorate reagent were also prepared for every analysis and treated in the same manner as the standards. These were necessary so that contamination of the reagent by absorption of ethylene present in the room air could be measured and subtracted from all of the samples.

A concentrated stock solution of ethylene was prepared by slowly bubbling reagent quality ethylene into a small quantity of mercuric perchlorate for two to four hours. The ethylene content of a 1/500 dilution of this concentrated stock was determined manometrically by the method of Young, Pratt and Biale (99) as described by Chandra (97). Values varying from 15 to 20 μl of ethylene per ml were obtained in different preparations. The calibrated stock solution was diluted a hundred fold and used in the prep-

aration of the standards. Both dilutions were accomplished with freshly prepared mercuric perchlorate reagent. Stock solutions were stored at 5°C to minimize deterioration.

c) Injection of Samples:

The gas samples were injected into the chromatographic column with a Hamilton "gas tight" syringe equipped with a Fisher and Burpe No. 25G hypodermic needle. However, prior to injection of the standards and unknowns, a strict sampling procedure was followed.

- i) penetration of hypodermic needle in gaseous contents of bottle

- ii) pumping of syringe plunger several times

- iii) withdrawal of 1.0 ml of gas sample

- iv) careful removal of syringe so as not to allow room air to seep into bottle.

After each injection the syringe was flushed with air several times to remove any trace of ethylene. During the course of the analysis it was important to check the needle for imbedded pieces of rubber. The latter can impede the flow of sample and at times the block may force the sample past the needle and its adaptor on the syringe. In addition, the interior of the needle was kept free from traces of mercuric perchlorate. Syringes were cleaned inside the barrel with tissue after every 10 to 15 injections to insure a tight fit between plunger and barrel wall.

d) Sensitivities Employed and Limit of Analysis:

Three sensitivity ranges on the Sargeant recorder were selected, namely; 25, 50 and 125 mV. The first two values

were used to determine the ethylene content of samples from the collection periods of 0-1, 1-3, 3-22 and 23-24 hr. Of these two recorder values the one that gave a pen deflection of approximately 100 divisions (chart paper of 250 divisions) with a standard of 0.30 to 0.40 μ l of ethylene was selected. The choice of ranges was an advantage in dealing with the variations in sensitivity of the chromatographic unit. The low sensitivity range of 125 mV was exclusively used in the analysis of samples from the 22-23 hr collection period. During the latter collection period a large quantity of ethylene is produced by the resonicated particles (41). A series of standards was run at each sensitivity range in order that calibration curves be drawn for computation of ethylene content in the samples.

The limit of sensitivity with a stable base line was 5 μ l of ethylene per ml of sample and quantities less than this were considered as trace amounts.

e) Calculation of Ethylene Content:

For each analysis calibration curves were drawn by plotting the peak heights of standards versus their ethylene content in μ l. The unknown samples were then evaluated from the curves and their values multiplied by a factor of 1.125 since only 4.0 ml of the total sample of 4.5 ml was determined.

f) Nitrogen Determinations:

The nitrogen content of each sample was determined by the microkjeldahl method of Street, Kenyan and Watson

(102) as outlined by Chandra (97). Nitrogen contributions from cofactors, substrates and inhibitors were subtracted from the gross total.

Although ethylene production can be expressed on a total nitrogen basis it was felt that many variables made such a basis questionable. The subcellular system is heterogeneous in nature and sources of nitrogen that have no influence on ethylene production would, therefore, be included in the determination. Moreover, it is not unreasonable to believe that ethylene biosynthesis be dependent on factors not associated with nitrogen content. Therefore, the yields of ethylene were not expressed as $\mu\text{l}/\text{mg}$ nitrogen but were recorded as $\text{m}\mu\text{l}$. The stated nitrogen values of each sample are used to indicate the uniformity of division of the suspension among the two or three samples in each experimental run.

g) Gas Chromatographic Unit:

The chromatograph constructed by Chandra (97) is based on a general design by McWilliam and Dewar (101) as described by Meigh (69) and basically is a dual flame hydrogen ionization type unit.

Two types of columns were used during the course of the experimental work. The first, a squalane impregnated firebrick column (97), gave a retention time of 25 to 30 seconds for ethylene when flow rates of 15 ml/2.6 sec for each of hydrogen and nitrogen were used. The second was a Burrel activated alumina (with 2 1/2% silicone 550) column packed into a 2 foot steel tube of 5/16" ID. A reten-

tion time of 12 seconds for ethylene was obtained with the same flow rates. The second column replaced the first one by virtue of its superiority in the separation of low molecular weight hydrocarbons.

A Sargent MR recorder was connected to the amplifier and geared to operate at a slow chart speed.

Voltage to the unit was controlled by a Raytheon 500 watt voltage stabilizer.

4. Procedures Used in Various Studies:

a) Study of Cofactors, Substrates and Inhibitors:

The effects of cofactors, substrates and inhibitors were evaluated by comparing the production of ethylene with that from a control sample made from the same preparation of subcellular particles, but lacking the particular constituent being tested. Whenever two or more factors were being studied concomitantly, the control sample lacked all of these constituents. Two examples may be cited to illustrate these procedures. The effect of adenosine triphosphate (ATP) would be determined by comparing ethylene production from the vessel containing the ATP cofactor with that of the vessel lacking it. The effect of ATP and nicotinamide adenine dinucleotide (NAD) would be determined by comparing ethylene production from the flask containing both constituents with that of the control vessel lacking both.

The concentrations used in all of the above studies were based primarily on the results reported by numerous workers on their research with subcellular systems (infor-

mation of this nature was located in the Annual Reviews of Biochemistry and of Plant Physiology and from the more prominent biological journals).

Almost all of the experimental runs contained a common cofactor, adenosine triphosphate. Since Chandra and Spencer (98) noted that ATP stimulated ethylene production in this tomato particulate fraction, the addition of an exogenous source of energy was adopted.

b) pH Studies:

The subcellular particles used in the pH studies were isolated by the usual procedures. Equal quantities of the particles were added to beakers of sucrose phosphate buffer (see 2a), one buffer adjusted to the usual pH value of 7.2 and the other adjusted to the value being studied. Dispersion was allowed to take place and each treated as a typical sample, as described in section 2 of this chapter.

c) Ageing Studies:

Several experiments were conducted with those substrates and cofactors that stimulated ethylene production significantly with particles that had not been disrupted initially by sonication. The particles in the presence of the added constituents were allowed to age overnight before they were sonicated. The purpose behind these experiments was to see whether the added constituents could induce accelerated ethylene evolution by intact particles and also observe whether there were any obvious differences in behavior of particles sonicated twice and

those sonicated once.

5. Additional Experimental Controls:

a) Sonication Controls:

In view of Meigh's proposal (69) that the sonication procedure itself could generate free radicals leading to the formation of ethylene, a series of sonication blanks was carried out with several substrates and cofactors. The intention of the study was to determine the extent, if any, of ethylene formation from the sonication of these factors, in the absence of subcellular particles. Since the cofactors and substrates were not sonicated initially but only after the ageing of the suspension, the procedure followed was to allow them to age for 22 hrs in 25 ml of buffer, sonicate the solution for 4 minutes at 1.2 amperes and then collect any ethylene for the next two hours. Quantitative production was the difference between the value of the sonicated factor and that obtained by sonication of the buffer.

b) Ethanol Controls:

A recent paper by Burg and Burg (58) suggested that ethylene could be formed by the dehydration of ethanol in mercuric perchlorate. Their results indicated a linear conversion in the first six hours, but only 0.02% of the ethanol was dehydrated. Before dehydration of ethanol could be regarded as a potential source of ethylene, steps were taken to verify the presence of ethanol in the volatiles being emitted. Mass spectrometry was one method

utilized in the identification tests for ethanol. The system used to collect the volatiles was arranged as follows:

i) purified air flushed the volatiles from the reaction vessel into a spiral condenser immersed in an acetone-liquid air slush (-95°C) to remove most of the CO_2 .

ii) the air stream was then passed into a U-tube containing activated alumina (Burrel No. 341-35) and immersed in liquid air.

iii) the volatiles collected in the U-tube were then submitted to a mass spectrometric analysis.

In this manner, volatiles having a boiling point higher than -147°C were trapped in the alumina. Ethanol freezes at -117°C and thus would be among the volatiles collected. Activated alumina was used in the collection of the volatiles because of its high absorptive capacity and large surface area. A sketch of the mass spectrogram for these volatiles is presented in Figure 1 on page 161.

A quantitative estimation of the ethanol content present in the particulate suspension at various times over a 24 hr. period was made by use of Ethanol Kit No. 330 from Sigma Chemical Company, St. Louis, Missouri, U.S.A. The alcohol content of a sample is calculated from the optical density of NADH read at 340 m μ . NADH is formed from the reduction of NAD by alcohol dehydro-

genase. Alcohols other than ethanol would be measured by the Sigma method but their contribution to the total ethanol content would be negligible because trace amounts of them would be expected in the particulate suspension. Furthermore, the ethanol evolved as a vapour by the tomato subcellular fraction was determined by collecting the volatiles in water and then quantitatively measuring the alcohol content. For the latter experiment volatiles were flushed from the reaction flask through a fine, porous glass filter into 5.0 ml of deionized water. Aliquots designated by the Sigma directions were then tested for their alcohol content. It should be stated that the particulate fraction was treated as a typical sample and thus, subjected to the same experimental procedure outlined in section 2. Results for the ethanol content of the particulate suspension and the volatiles are given in Tables X and XI, respectively.

The possible contribution of ethylene from the dehydration of ethanol in mercuric perchlorate to the total ethylene measured was evaluated by allowing ethanol to age in the reagent. The amount of ethanol used in these studies was determined by the methods described in the preceding paragraph. Following the liberation procedure stated in 3(b), the samples were subjected to gas chromatographic analysis for the detection of ethylene. Samples were aged for 24, 48, 72 and 96 hours, respectively, a period representing the storage time for samples collected from the particulate suspension. The results of ethylene

formation from ethanol dehydration are discussed on Page 158.

6. Confirmation of the Identity of Ethylene:

Periodic checks were made to validate the fact that the volatile being studied was ethylene. Two methods were utilized in the identification of the olefin, namely, absorption in bromine water and mass spectrometric analysis.

a) Absorption by Bromine Water:

In this method duplicate samples of an ethylene standard and an unknown were required. The ethylene in the 4.0 ml samples, kept in stoppered serum bottles described in section 2, was liberated by the usual procedure. Stoppered serum bottles containing 5.0 ml of saturated bromine water were also prepared and 1.0 ml of the gaseous atmosphere in each vial was removed with a syringe. Then 1.0 ml gas samples from a standard and an unknown were injected into separate vials containing the bromine water. The vials were immediately shaken for several minutes. Next 1.0 ml gas samples from the intact standard and unknown samples and the bromine water samples were injected into the gas chromatograph. The appearance of peaks at the specific retention time for ethylene on the chromatogram for the standard and the unknown, and the disappearance of peaks for the bromine water treated samples was an indication that ethylene was present in the unknown sample.

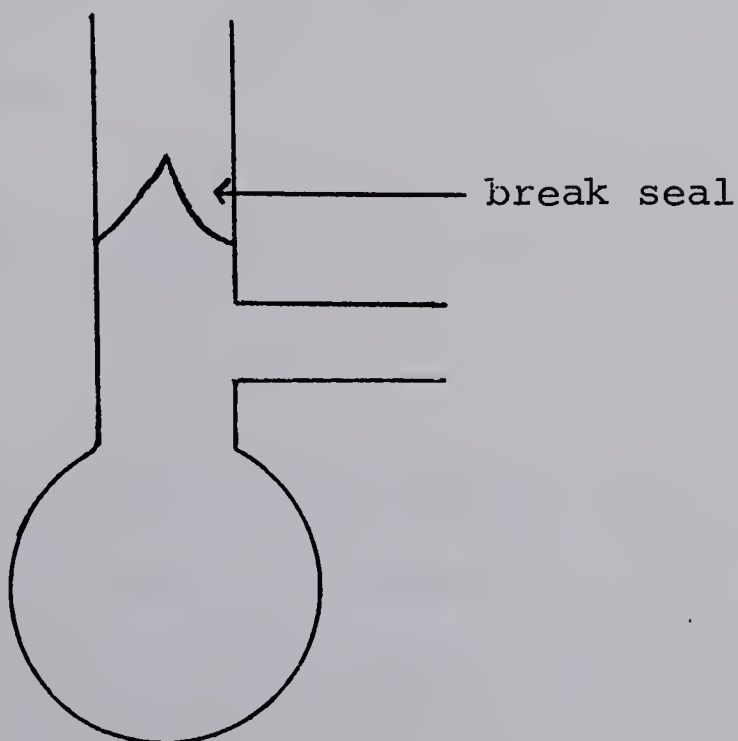
b) Mass Spectrometry:

Two methods were used in the mass spectrometric

analysis. The collection and liberation of the samples were conducted in the manner described in section 2.

In the first method, a 4 inch section of 10 mm glass tube closed at one end, with a short side-arm inlet near the top equipped with a serum cap, was connected to the mass spectrometer and evacuated. A dewar flask containing an isopentane-liquid nitrogen slush was elevated to submerge the tube. The gas sample was then introduced through the serum cap into the tube and allowed to equilibrate with the temperature of the slush, -160°C . At the end of several minutes the gas sample was pumped into the instrument for analysis. Vapour pressures of ethylene and carbon dioxide at -160°C are 3 mm and $\ll 1$ mm respectively. Since both gases give a peak at 28, the low vapour pressure of carbon dioxide would, therefore, result in a minimum of interference.

The second method consisted of constructing a "break seal" unit of the type below.



Total capacity of the unit was approximately 10 ml. The mercuric perchlorate solution containing the ethylene complex was introduced into the bulb and frozen in an acetone-dry ice bath. Then 1 ml of 4 N LiCl was carefully layered on the perchlorate reagent and frozen immediately. The entire tube was evacuated through the side-arm to a pressure of 0.1 Hg and the side-arm carefully sealed with a flame. The unit was allowed to come to room temperature and liberation of the olefin followed. It was then connected to the mass spectrometer, the neck evacuated, the seal broken, and finally a sample withdrawn for analysis.

Typical spectrograms of the volatile are indicated in Figures 2 and 3 (pages 166 and 167, respectively).

Effects of Various Substrates on the Production of Ethylene

As yet no pathway has been conclusively established for the biosynthesis of ethylene. Tracer studies, which are often used in the verification of the sequential steps of a postulated pathway, have yielded only limited success in the elucidation of a pathway for ethylene. The labelling of ethylene by $[C^{14}]$ substrates has, on the whole, been too weak to permit the identification of a major precursor to ethylene. Tracer studies by three laboratories have culminated in the proposal of three pathways for the biosynthesis of ethylene. The first, suggested by Wang, Jacobsen and Tanaka (54) is dependent upon the TCA cycle.

(1) Succinate→fumarate→malate→acrylate→ethylene

The second pathway utilizes β -alanine as a major intermediate and was proposed by Thompson and Spencer (74).

(2) β -alanine→malonic semialdehyde→ β -hydroxypropionate→acrylate→ethylene

The third pathway, dependent upon glycolysis, was proposed by Burg and Burg (103).

(3) Glucose $\begin{matrix} \nearrow \text{glycolysis} \\ \searrow \text{oxidation} \end{matrix} \rightarrow \text{pyruvate} \rightarrow \text{acetate} \rightarrow \text{fumarate} \rightarrow ? \rightarrow \text{ethylene}$

Acrylate appears as an immediate precursor to ethylene in the first two pathways and may be the unknown intermediate in the pathway of Burg and Burg (103). The latter assump-

tion is acceptable in view of the labelling of ethylene by fumarate in pathways (1) and (3). The importance of the three pathways with respect to the results obtained when various substrates were added to a tomato particulate fraction will become apparent in the results and discussion.

Numerous substrates could be evaluated as potential precursors to ethylene but if random selection were followed research of this nature would be lengthy and probably unrewarding. The substrates studied by the author were suggested by several sources; published papers of other workers, on the subject of ethylene, the author's personal research, and knowledge of some of the biochemical events in fruit ripening (104). The observations by Hulme (105, 106) and Rowan, Pratt and Robertson (107) that net protein content increased during the climacteric period of apples suggested the use of aspartic and glutamic acid. The amino acids were chosen in preference to others because they are not only more prevalent in tissue but actively participate in numerous reactions. Besides those mentioned, other substrates evaluated included an important intermediate from some major metabolic pathways and also one or two intermediary products of substrates found to be effective in ethylene production.

The complex enzyme system residing in the particulate fraction coupled with the highly regulated process of ethylene production requires an interpretation of results that is both challenging and frustrating. The significance of the statement will become apparent in a

moment. Lack of an effect does not warrant the conclusion that a substrate is ineffective as a precursor to ethylene since several factors may influence its behavior. An endogenous supply of the substrate may already be present and as a result, the exogenous source may not have any effect on ethylene production. Many enzyme reactions are rate limiting and addition of a substrate whose metabolism is governed by such an enzyme may result in weak stimulation of ethylene evolution or none at all if the substrate is already present in the particulate fraction.

Quite often cofactors are necessary for metabolism of substrates. This was evident with studies concerning β -alanine. Loss of cofactors by diffusion from the particles during the isolation procedure could certainly depress the metabolism of some substrates.

How the soluble phase of the cell is integrated with the particulate fraction in regards to ethylene biosynthesis is unknown. Metabolism of some substrates to ethylene may be profoundly affected by disruption of this integration. Furthermore, enzymes responsible for metabolism of some potential precursors to ethylene may reside in the soluble phase only.

The pH of the medium may have a pronounced effect on the metabolism of substrates. This would be especially relevant to optimum pH values of specific enzymes.

Some substrates may act through a feedback mechanism. Initiation of a feedback mechanism may open new avenues of metabolism not conducive to ethylene biosynthesis or it may result in the synthesis of compounds

inhibitory to enzymes concerned with ethylene biogenesis.

Still another consideration is that some substrates at the concentration used may be inhibitory to enzymes associated with ethylene production. Other conditions may certainly govern ethylene production but those mentioned will, no doubt, allow the reader to appreciate the difficulty in attempting to interpret the lack of an effect or the appearance of an unusual effect with any of the substrates used. In spite of the difficulties listed several advantages are evident in the use of a particulate fraction for studying the biogenesis of ethylene. Since fewer metabolic pathways are present in the particulate fraction than in whole tissue determining the likely metabolic fate of an added compound is, therefore, facilitated. A certain degree of organization of the enzymes would remain in the particulate fraction but would be absent in a purified enzyme preparation. In this respect, a close resemblance to in vivo conditions would be evident with the particulate fraction. Moreover, some reactions observed with an enzyme preparation may have no relevance to in vivo biochemical events or they may not occur at all in whole tissue. These reactions probably would not occur in a particulate fraction because biochemical reactions normally are strictly regulated in an organized complex of enzymes. In all, the particulate fraction from tomatoes offers an enzyme system less complex than present in whole tissue yet more extensive and organized than that of a purified enzyme preparation.

The evaluation of the effects of the substrates studied is that on a fragmented particulate fraction from ripening tomatoes and does not necessarily indicate similar behavior in whole tissue or intact particles. However, the purpose at hand is not to demonstrate a similarity but rather to elucidate a biosynthetic pathway for ethylene in a simplified system. The pathway can then be applied to in vivo studies and authenticated, altered or rejected.

Results and Discussion:

The results obtained from the studies of the effects of substrates are given in Table I (pages 49-53). It must be stressed that results for any substrate must be considered as qualitative in nature. This is necessary because of the inability by any techniques known at present to isolate subcellular particles of the same quantitative yield and biochemical properties. Nevertheless, valid comparisons may be made on a quantitative basis within runs and on a qualitative basis between runs.

Although only two runs are reported in the tables for most of the individual experiments, these represent typical results from several runs that were completed. Whenever results were found to be inconsistent the two selected were ones that were indicative of the inconsistent behavior.

Bacterial contamination is a distinct possibility in any of the runs but any contribution to the total yield of ethylene is apparently negligible. The assumption is based on the lack of significant ethylene production during the 0-22 hr. ageing period for the initially intact particles as described in Chapter 11. However, changes in the composition of the reaction mixture may have occurred but how they may have affected the behavior of the particles with regards to the evolution of the volatile is unknown.

a) Glycolaldehyde (0.05 M) : Runs 1, 2

Total ethylene production and that from the aged, resonicated particles was markedly depressed by the addition of glycolaldehyde.

The pronounced inhibitory effect of glycolaldehyde may be explained by three modes of action. First, it may have reacted with most of the thiamine pyrophosphate to form hydroxyethylethiamine pyrophosphate, otherwise known

as "active glycoaldehyde". If the complex were not acted upon for lack of the appropriate enzyme then cocarboxylase required for decarboxylation would be unavailable. Support for decarboxylation stems from the stimulation of ethylene production by cocarboxylase and magnesium (see Chapter 4). Secondly, glycolaldehyde may participate in the formation of the inhibitor, α -hydroxyoxalsuccinate, by its conversion to glyoxylate and subsequent condensation with oxalacetate. The inhibitor was first reported by Ruffo, Testa, Adinolfi and Pelizza (108) to suppress aconitase activity. Later Payes and Laties (109) stated that α -hydroxyoxalsuccinate was decarboxylated to γ -hydroxyglutarate, an inhibitor found to inactivate aconitase, isocitric and α -ketoglutaric dehydrogenase. Glycoaldehyde oxidation to glyoxylate has been reported in plants and animals (110, 111, 112). The third mode of action may be that of its effect on α -carboxylase. Juni (113) observed inhibition of yeast α -carboxylase by several aldehydes, one of which was glycolaldehyde.

Of the three modes of action described the one that seems most applicable to glycolaldehyde is its effect on α -carboxylase. The reasons for this selection are two, first, the stimulatory effect of cocarboxylase and Mg^{++} indicates potential carboxylase activity and second, the weak inhibition given by glyoxylate suggests low formation of the inhibitor, γ -hydroxyglutaric acid.

b) Glucose (0.05 M): Runs 3, 4

Glucose exhibited little effect on ethylene produc-

tion by the tomato particulate fraction.

It is generally believed that the complete roster of enzymes of the glycolytic pathway (EMP) and the hexosemonophosphate shunt (HMP) reside in the soluble phase of the cell and therefore, the tomatoe particulate fraction would not be expected to metabolize glucose to the end product of each pathway unless residual traces of all the enzymes were isolated with the fraction. Abood (114) reported that rat brain mitochondria contained all the glycolytic enzymes and he suggested that they were bound to the surface of the particle. However, the author has not encountered additional papers to corroborate the observation. To what extent glucose is metabolized in the particulate fraction from tomatoes is unknown but if metabolic products of glucose do occur they appear to have little effect on ethylene production.

Glucose has been reported to support ethylene production in Penicillium digitatum (40, 51) and administration of labelled glucose has resulted in weakly labelled ethylene evolved by the fungus (40, 53). Apple tissue labelled ethylene significantly when supplied with glucose-6-C¹⁴ (57), a result also observed with Penicillium digitatum (53). The weak labelling of ethylene by labelled carbon from glucose indicates that it does not act as a major precursor to ethylene but is a distant precursor slowly metabolized to ethylene, probably according to the scheme outlined by Burg and Burg (103).

c) Galactose (0.05 M): Runs 5, 6

The production of ethylene for the 22-24 hr. period was stimulated significantly in the presence of galactose.

The augmentation in ethylene production by galactose is not a result of its epimerization to glucose because the latter carbohydrate had no influence on evolution of the gas. The result is complicated by the fact that enzymes responsible for the metabolism of galactose are located in the soluble phase of the cell. However, the article by Isherwood and Mapson (115) that galactose can be converted to ascorbic acid by a combination of enzymes from the soluble phase and a particulate fraction offers a possible explanation for the stimulation by galactose. How the formation of ascorbic acid would influence ethylene production other than by a protective role as an anti-oxidant is unknown.

Galactose was found to sustain ethylene production in Penicillium digitatum (49, 50) and like glucose, the sugar may be slowly converted to ethylene in whole tissue.

d) Xylose (0.05 M): Runs 7, 8

Initial production of ethylene was increased by xylose but the total production was only slightly higher than that of the control.

The lack of a stimulatory effect with xylose agrees with the tracer studies of Burg and Thimann (57) in which no label appeared in ethylene from xylose-¹⁴C. The tomatoe particulate fraction would not be

expected to metabolize xylose because the necessary enzymes are found in the soluble phase of the cell. The result by Fergus (49) that Penicillium digitatum evolved ethylene from a xylose carbon source raises the question of whether different pathways exist in the fruit and fungus for the biogenesis of ethylene.

Xylose apparently does not occur in the free state (116) and thus, would be unavailable in the fruit as a potential precursor to ethylene.

e) Ethanol (0.05 M): Runs 9,10

A pronounced increase in production of the olefin was given by ethanol and increases exceeding 100% were recorded for the 22-24 hr. collection period.

Ethanol has been observed by other workers to be an effective precursor to ethylene. Phan Chan Ton (40) on the basis of his results with Penicillium digitatum postulated ethanol to be an immediate precursor to ethylene. Hall (50), too, found ethanol to be effective in promoting ethylene production by the fungus. Tracer studies with the fungus by Gibson (52) revealed a 1.55% uptake of label by ethylene from ethanol-2-C¹⁴ and 0.35% from ethanol-1-C¹⁴. Plants normally produce ethanol (117) and it has been shown to be metabolized primarily to acetyl.CoA (118). Since acetyl.CoA can enter into numerous reactions it would be difficult to know whether the acyl compound proceeds to ethylene by entry into the TCA cycle or by conversion to a compound that undergoes degradation to the volatile by some

other pathway. A dehydration of ethanol to ethylene would coincide with Burg and Thimann's postulation (30) that an intermediate capable of reversible dehydration yielded a compound readily converted to ethylene. A result contrary to the ones above was reported by Abeles and Rubenstein (73) as no stimulatory effect on ethylene production from ethanol by a pea enzyme preparation was observed.

f) Propionic Acid (0.05 M): Runs 11, 12

Both the initial production and that from the aged resonicated particles was markedly increased by propionic acid.

Propionic acid usually results from the β -oxidation of odd numbered carbon fatty acids and alpha branched amino acids. Labelling of ethylene by propionate has been reported by Burg and Burg (58). Ethylene biosynthesis from propionate can be realized from the pathways proposed by Thompson and Spencer (74) and Wang et al (54) provided that propionate is converted to acrylate. In fact the formation of acrylate or acrylyl.CoA from propionate has been reported by Giovanelli and Stumpf (119, 120), Mahler and Huennekens (121), Rendina and Coon (122), Stadtman (123) and Stumpf (124). Metabolism of propionate to pyruvate (121) probably would not result in stimulation of ethylene production, since pyruvate was found to be ineffective as a precursor. However, the carboxylation of propionate to methylmalonate and its subsequent

isomerization to succinate (125, 126) would yield fumarate, a compound known to label ethylene (52, 54, 103).

A cofactor requirement for propionate metabolism is CoA (119) but ethylene production from a suspension containing propionic acid and CoA was lower than that of a control containing the acid but not the cofactor. The addition of CoA may result in the formation of propionyl-CoA, a compound that may not proceed to ethylene or a reaction may be mediated by the cofactor that interferes with ethylene synthesis.

g) Pyruvate (sodium salt) (0.05 M): Run 13, 14

Production of ethylene for the 22-24 hr. period decreased appreciably in the presence of pyruvate.

Fergus (49) found pyruvate to be ineffective as a source of ethylene with Penicillium digitatum but Gibson (127), Hall (50) and Spalding and Lieberman (51) all reported production of ethylene from pyruvate by the fungus. Gibson (52) noted pyruvate-2-C¹⁴ to be better in labelling of ethylene than was pyruvate-1-C¹⁴, in Penicillium digitatum, and concluded from the results that ethylene was derived from a two carbon fragment not far removed from pyruvate. Burg and Burg (103) have suggested pyruvate as an intermediate in the pathway of glucose to ethylene. Pyruvate may have been inhibitory to ethylene production by the tomato particulate fraction by virtue of its metabolism to an intermediate detrimental to the production. Formation of acetaldehyde, for instance,

would inhibit the activity of α -carboxylase.

h) α -Ketoglutaric acid (0.05 M): Runs 15, 16

Total ethylene production increased slightly when α -ketoglutaric acid was added to the tomato particulate fraction.

This intermediate of the TCA cycle has not been evaluated by other workers and from the low stimulation of ethylene production initiated by it one might presume that this step of the TCA cycle is not important to ethylene production. However, the observation by Linnane and Zeigler (128) that disruption of the TCA cycle occurs in disintegrated mitochondria may be significant to α -ketoglutaric acid metabolism. Enzymes of the TCA cycle apparently were solubilized when mitochondria were sonically treated. Consequently, whether α -ketoglutaric acid is metabolized extensively in this tomato preparation is unknown but if succinate were formed it would appear to be ineffective as a substrate for conversion to ethylene. Both Gibson (52) and Wang et al (54) found succinate to be weak in labelling ethylene and it is conceivable then that succinate is not a significant precursor of ethylene.

Other TCA cycle intermediates found to be effective in promoting ethylene production are citrate (49, 151, 127), fumarate (54, 58, 129) and malate (40, 49, 51, 127). Wang et al (53) detected labelled ethylene when alanine, aspartic and glutamic acid were administered to Penicillium digitatum and the basis of the re-

sults concluded that the TCA cycle was directly involved in the biogenesis of ethylene. Tracer studies that partially support the conclusion are those of Gibson (52) and Burg and Burg (58) in which fumarate and succinate, and fumarate, respectively, labelled ethylene.

The extent of the TCA cycle necessary to ethylene production in the tomato particulate preparation cannot be stated unequivocally but the lack of significant inhibition of ethylene evolution with malonate and monofluoroacetate (see Chapter 5) suggests that a considerable portion of it is not essential.

- i) Aspartic acid (0.01 M and 0.05 M): Runs 17, 18, 19, 20

A large increase in the total yield of ethylene was realized with the higher level of aspartic acid. However, when the concentration was reduced to 0.01 M the increase was only 15%.

Very little emphasis has been directed to the observation that net protein content increases during the climacteric period of fruits (105, 106, 107), and amino acids have received limited evaluation as potential precursors to ethylene. Phan Chan Ton (40) detected no label in ethylene from aspartic acid but contrary results were obtained by Wang et al (53). Penicillium digitatum was used in both studies.

Entry of aspartic acid into the TCA cycle as oxalacetate would be possible after transamination with α -ketoglutarate or after oxidative deamination. In

order that oxalacetate proceed to ethylene via the pathways of Burg and Burg (103) and Wang et al (54) an organized TCA cycle would be necessary. Since the cycle is disrupted in fragmented mitochondria (128) it would be more reasonable to assume a reduction of oxalacetate to malate. Perhaps the significance of the greater stimulation of ethylene evolution with the higher concentration of aspartic acid is enhanced malate synthesis from oxalacetate.

Decarboxylation of aspartic acid to give β -alanine is known in bacteria (130, 131, 132) but has not been reported as yet in plants (133) and, therefore, cannot be used as a possible explanation for the marked increase in ethylene produced caused by aspartic acid.

j) Glutamic acid (0.05 M and 0.01 M): Runs 21,
22, 23, 24

Ethylene production was greatly elevated when 0.05 M glutamic acid was added to the particulate fraction. Decreasing the concentration of glutamic acid to 0.01 M resulted in an increase considerably less than that obtained with the higher level of the amino acid.

The similar behavior of aspartic and glutamic acid suggest a close relationship in their metabolism to ethylene or influence on ethylene production. Tracer studies with glutamate injected into Penicillium digitatum resulted in labelling of ethylene for Wang et al (53) but not for Phan Chan Ton (40). Glutamate was reported to stimu-

late ethylene production in bacteria (129). The conversion of glutamate to α -ketoglutarate by transamination with oxalacetate or pyruvate is an unlikely basis for the stimulation of ethylene evolution, especially in view of the ineffectiveness of α -ketoglutarate as a precursor of ethylene. If aspartic acid were metabolized to ethylene by a pathway other than by the suggested conversion to oxalacetate then the stimulatory influence of glutamate on ethylene production may be a result of its transamination with oxalacetate to form aspartate.

The decarboxylation product, γ -aminobutyric acid will be discussed separately but the results indicate its formation is not the entire mode of action for glutamic acid. Catabolism of glutamate to pyrrolidine carboxylic acid (134), normally a precursor to proline is doubtful as a stimulatory agent of ethylene evolution. The observation by Smith et al (135) that glutamate sparked propionate metabolism in sheep liver mitochondria by acting as an energy supply suggested an analogous effect with the tomato particulate fraction. However, a potential source of energy is doubtful as the sole function of glutamic acid. One might expect similar yields of ethylene from both concentrations of the amino acid since they both probably exceed the physiological levels. Another expectation might be a five fold difference in the yield of the olefin between the two levels of glutamic acid but only a three fold difference was observed. A high adenosine triphosphatase activity or rate limiting enzyme reactions would, however, reduce the

possibility of achieving the five fold increase in ethylene production. The fact remains that an equilibrium conducive to ethylene production is initiated by the higher concentration of aspartic and glutamic acid and perhaps metabolic roles exclusive of the TCA cycle exist for the conversion of these amino acids to ethylene.

k) γ -Aminobutyric acid (0.05 M) : Runs 25, 26

Overall production of ethylene increased in the presence of γ -aminobutyric acid.

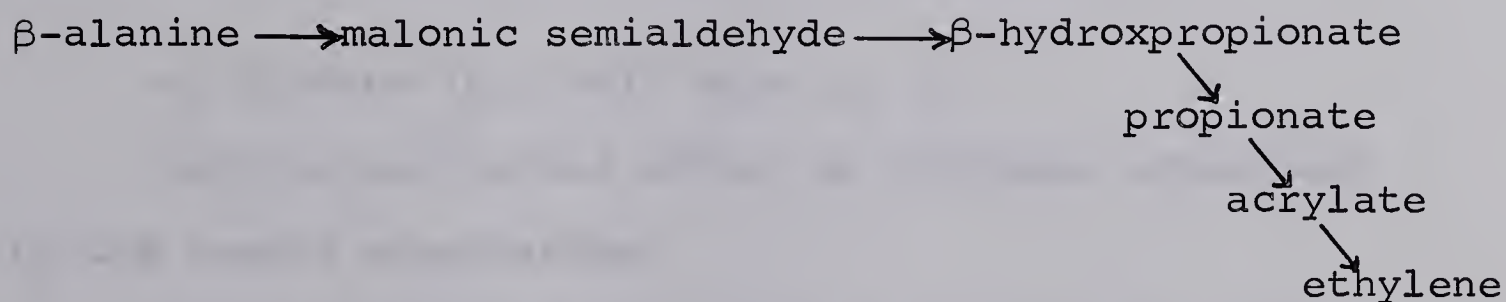
Decarboxylation of glutamic acid to γ -aminobutyric acid is well known (136, 137, 138). The product undergoes transamination with α -ketoglutaric or pyruvic acid to form succinylsemialdehyde, which is then converted to succinate (139, 140, 141). The formation of succinate may explain the weaker stimulation of ethylene production by γ -aminobutyric acid compared to glutamic acid, provided that labelling of ethylene by succinate (40, 54, 58) can be used as corroborating support. More likely, though, is the formation of aspartic acid from the transamination of oxalacetate with γ -aminobutyric acid. Thus the latter amino acid may be less effective than glutamic acid because the rate of formation of aspartic acid from it is lower than from glutamic acid.

l) β -Alanine (0.05 M) : Runs 27, 28

Only a small increase in the yeild of ethylene was recorded with β -alanine.

The importance of β -alanine to ethylene biosynthesis

was suggested by Thompson and Spencer (74). In tracer studies label from β -alanine was incorporated into ethylene and tentative intermediates in the pathway were also found to contain labelled carbon. β -alanine has been detected in plants and animals (142, 143) and arises in plants by degradation of nucleic acids (144, 145). Some of the metabolic products formed by the metabolism of β -alanine are acetyl.CoA (146), malonic semialdehyde (147) and propionate (148). Propionate formation is significant because the fatty acid was found to be an effective precursor of ethylene. In fact, it is conceivable that propionate is an intermediate in the conversion of β -alanine to ethylene. This seems reasonable in view of the greater stimulatory effect of propionic acid over that of β -alanine on ethylene production by the tomato particulate fraction. The pathway for β -alanine metabolism to ethylene might be as follows;



Wang et al (54) have also detected labelled ethylene from β -alanine in Penicillium digitatum and therefore, affirm the observations of Thompson and Spencer with an enzyme system from bean seedlings. At this point, it should be mentioned that β -alanine metabolism to ethylene is stimulated by addition of certain cofactors. A

complete study of the effects of cofactors on β -alanine metabolism is presented in Chapters 8, 9 and 10.

m) Serine (0.05 M): Runs 29, 30

Serine induced an increase of 25% in total ethylene production by the tomato particulate fraction.

The weaker effect of serine compared with glutamic acid may suggest a different metabolic role for the amino acid in ethylene evolution. Degradation of serine usually results in the formation of pyruvate, glycine or cysteine. Pyruvate was found to inhibit ethylene production and therefore cannot be considered as the agent responsible for the effect of serine. Cysteine, is not suspected because glutathione (see Chapter 4) decreased the yield of the volatile and both are thiol compounds. Glycine, which is discussed next, essentially had no effect on ethylene evolution. Participation in methionine metabolism is another role possible for serine. The likelihood of this occurrence is discussed later in the chapter.

n) Glycine (0.05 M): Runs 31, 32

Glycine had little effect on ethylene production by the tomato preparation.

Weak labelling of ethylene by glycine was reported by Wang et al (53) but Abeles and Rubenstein (73) found it to be ineffective as a substrate for ethylene in a pea enzyme preparation. Moreover, the deamination of glycine to glyoxylate seems doubtful because the latter compound inhibited production significantly.

o) Glyoxylate (sodium salt) (0.05 M): Runs 33,34

An appreciable decrease in the yield of the olefin was measured when glyoxylate was used as a substrate.

One basis for inhibition by glyoxylate is the formation of the TCA cycle inhibitor, γ -hydroxyglutaric acid (109). Another possible effect may be through the withdrawal of acetyl.CoA to form oxalacetate, an acid that may suppress production. Although malate has been implicated in ethylene biogenesis (40, 49, 51, 127), sufficient oxalacetate may be formed to nullify the conversion of malate to ethylene if the enzyme responsible for the latter conversion is sensitive to oxalacetate. Abeles and Rubenstein (73) observed no stimulation of ethylene production from glyoxylate by a pea enzyme preparation.

p) Acrylic acid (0.05 M):Runs 35, 36

Acrylic acid inhibited ethylene evolution by the tomato particulate fraction.

The inhibition by acrylic acid would suggest that it is not a precursor to ethylene. However, a number of factors may have affected the metabolism of acrylic acid to ethylene. The concentration of the acid used (0.05 M) may have been inhibitory to the enzyme that decarboxylates it to ethylene. There is, also, the possibility that other enzymes associated with ethylene biosynthesis may have been inhibited by acrylic acid. Since decarboxylation of acrylic acid is involved in the conversion of acrylate to ethylene, a lack of thiamine pyrophosphate and magnesium ions would have re-

duced the rate of the reaction considerably. Hopper and Segal (149) reported that acrylate inhibited a glutamic-alanine transaminase. While it is conceivable that a lower concentration of acrylic acid might be stimulatory to ethylene production, the acid is extremely reactive chemically, and much of it could be expected to enter other reactions before consumption as a substrate in ethylene production.

Although several substances were evaluated as potential precursors to ethylene only a few were demonstrated to influence ethylene production significantly. Effective ones were ethanol, propionic acid, aspartic acid, glutamic acid and serine. At this point the reader may ask whether a relationship exists for the metabolism of the substances to ethylene, assuming they act as precursors rather than in less direct ways. Establishing such a relationship is difficult unless results from tracer studies are available. However, a recent paper by Larson and Beevers (150) disclosed that pea seedlings contain homoserine as their major amino acid and that it is not present in ungerminated seeds. Tracer studies with glutamic and aspartic acid resulted in homoserine of high activity. These disclosures have assisted in the expression of a metabolic sequence for the conversion of the effective substances to ethylene. The fact that seedlings produce ethylene (18, 151) suggested an association between homoserine metabolism and ethylene biosynthesis.

Homoserine can be deaminated to α -ketobutyrate, (154), which subsequently can be decarboxylated to propionate,

an intermediate included in the proposed pathway for biogenesis of ethylene. Homoserine, therefore, will be used as a key intermediate in the suggested scheme for the biosynthesis of ethylene. Aspartic acid can be converted to homoserine via β -aspartylsemialdehyde (152). Transamination, which is highly active in seedlings (153), would give rise to aspartic acid from glutamic acid. Serine would participate in methionine catabolism to homoserine (154). Aspartase activity has also been included because it would yield fumarate, a compound shown to label ethylene (58, 54, 129). A scheme incorporating the results of other workers and those of the author's would appear as follows:

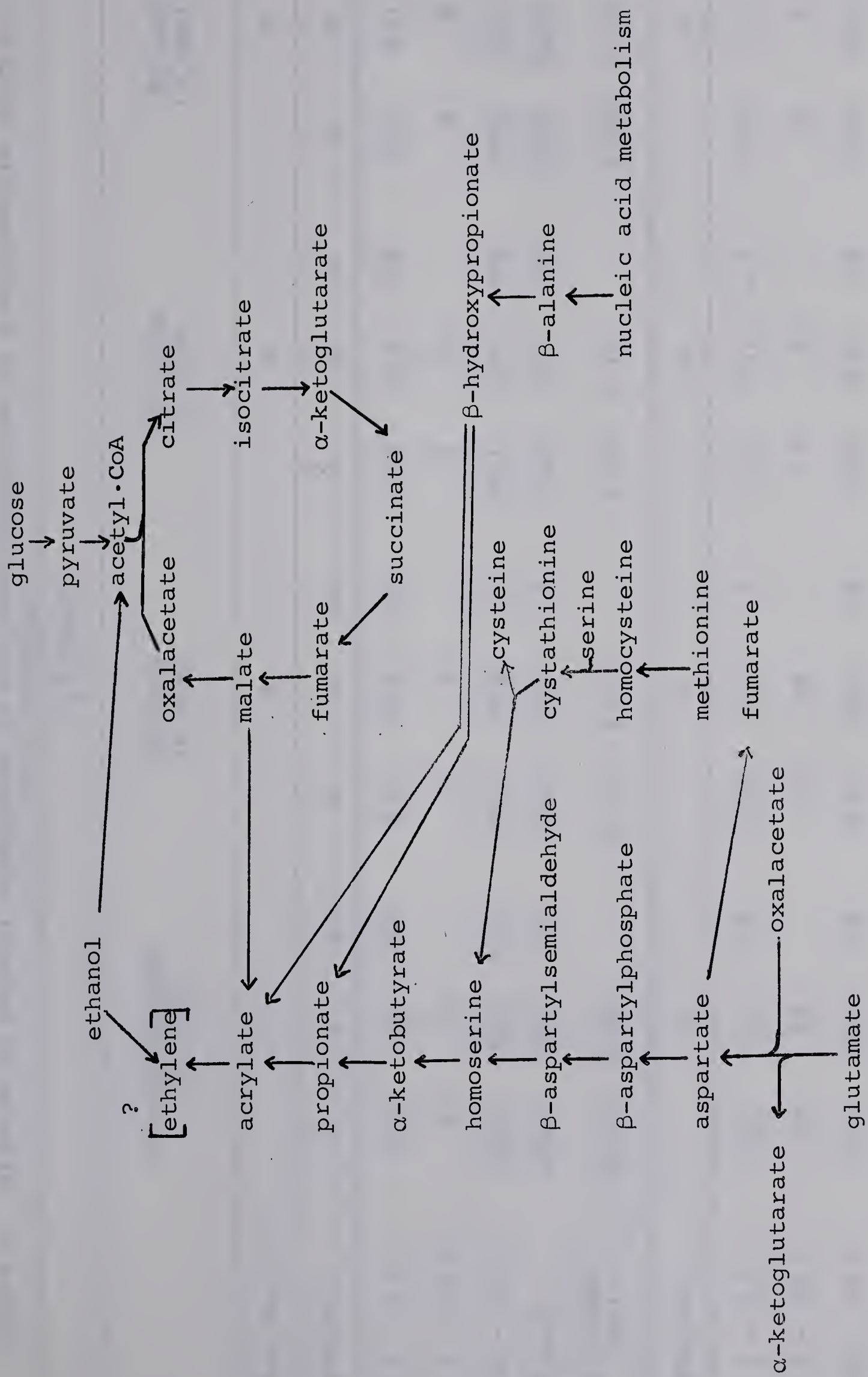


Table I. Effects of Several Substrates on Ethylene Production by a Particulate Fraction from Tomatoes

C ₂ H ₄ (μl)													
Glycolaldehyde (0.05M)				Glucose (0.05M)				Galactose (0.05M)				Xylose (0.05M)	
Run No.				3				5				7	
Period	A	B	%	A	B	%	A	B	%	A	B	%	%
0 - 3 h	259	93	-64	235	270	+15	338	276	-18	207	277	+34	
3 - 22 h	N	N		N	N		N	25		N	N		
22 - 24 h	3839	878	-77	861	928	+ 8	411	675	+64	895	851	- 5	
Total	4098	971	-76	1096	1198	+ 9	749	976	+30	1102	1128	+ 2	
Nitrogen (mg)	9.15	9.50		4.11	4.29		7.79	7.79		7.26	7.18		
6													
Run No.				4				6				8	
0 - 3 h	123	157	+28	343	301	-12	197	201	+ 2	207	309	+49	
3 - 22 h	39	14		73	84		17	N		N	N		
22 - 24 h	911	407	-55	1412	1226	-13	326	438	+34	895	1029	+15	
Total	1073	578	-46	1828	1611	-12	540	641	+19	1102	1338	+21	
Nitrogen (mg)	10.33	9.80		7.64	8.05		8.14	8.40		7.26	6.13		

Table I. (cont'd)

C ₂ H ₄ (mpul)															
Ethanol (0.05M)				Propionic Acid (0.05M)				Pyruvate (Na salt) (0.05M)				α-Ketoglutaric Acid (0.05M)			

Table I. (cont'd)

C ₂ H ₄ (m μ l)											
Aspartic Acid (0.05M)			Aspartic Acid (0.01M)			Glutamic Acid (0.05M)			Glutamic Acid (0.01M)		
Run No. 17			19			21			23		
Period	A	B	%	A	B	%	A	B	%	A	B
0 - 3 h	141	180	+28	141	73	-48	225	197	-12	332	248
3 - 22 h	N	N		23	N		N	23		27	N
22 - 24 h	5380	6649	+24	558	638	+14	647	1252	+94	1287	1783
Total	5521	6829	+24	722	711	- 2	872	1472	+69	1646	2031
Nitrogen (mg)	12.34	12.60		9.40	10.66		5.15	5.16		9.57	9.72
Run No. 18			20			22			24		
0 - 3 h	343	347	+ 1	293	326	+11	174	293	+68	110	152
3 - 22 h	73	23		101	96		104	38		45	45
22 - 24 h	1412	2757	+95	2807	3264	+16	1328	1895	+43	392	450
Total	1828	3127	+71	3201	3686	+15	1606	2226	+39	547	647
Nitrogen (mg)	7.64	7.72		7.79	7.58		8.23	8.52		6.83	7.00

Table I. (cont'd)

C ₂ H ₄ (mμl)											
γ-Aminobutyric Acid (0.05M)				β-Alanine (0.05M)				Serine (0.05M)		Glycine (0.05M)	
Run No.	25			27			29			31	
Period	A	B	%	A	B	%	A	B	%	A	%
0 - 3 h	358	332	- 7	117	97	-17	258	236	- 9	154	-19
3 - 22 h	62	T		17	T		27	27		9	
22 - 24 h	809	1141	+41	419	506	+21	1984	2756	+39	615	+12
Total	1229	1473	+19	536	603	+13	2269	3119	+37	678	+26
Nitrogen (mg)	7.74	7.09		7.42	7.42		10.59	9.05		8.66	9.19
Run No.	26			28			30			32	
0 - 3 h	203	219	+ 8	191	182	- 5	203	338	+67	230	-21
3 - 22 h	77	45		100	54		98	56		51	68
22 - 24 h	953	1325	+39	304	562	+85	2209	2552	+16	587	- 7
Total	1233	1589	+29	595	798	+34	2510	2946	+17	868	- 8
Nitrogen (mg)	5.46	4.48		5.99	5.63		9.28	8.31		7.00	6.96

Table I. (cont'd)

		(C ₂ H ₄ (m μ l)			
		Glyoxylate (Na salt) (0.05M)		Acrylic Acid (0.05M)	
Run No.		33		35	
Period		A	B	%	%
0 - 3 h		191	171	-10	+1
3 - 22 h		17	63	N	14
22 - 24 h		975	604	-38	-45
Total		1183	838	-29	-33
Nitrogen (mg)		7.61	7.79	7.79	6.48
Run No.		34		36	
0 - 3 h		176	163	-7	-8
3 - 22 h		15	36	73	28
22 - 24 h		638	478	-25	-12
Total		829	677	-18	-16
Nitrogen (mg)		10.90	10.99	6.48	7.00

Basic Reaction Mixture
0.5M sucrose
0.125M KH₂PO₄
pH 7.2
1.9 x 10⁻³M ATP added
to each flask after initial
sonication. Particles were
sonicated for 4 min. at 1.2
amp. at the beginning of
the 0-3 hr. and 22-24 hr.
collection periods, respectively.

A = control containing particles
suspended in basic reaction
mixture but without substrate
B = sample containing the same
as A but with added substrate
% = percent increase or decrease
in ethylene production
T = trace amount
N = none detectable

Chapter 4.

4. Effect of Several Cofactors on Ethylene Production

Comprehensive studies on the effects of cofactors on ethylene production by subcellular systems have been rather limited. Abeles and Rubenstein (73) published a preliminary note on the effects of several cofactors on ethylene evolution by a pea enzyme preparation. Subcellular fractions used by other workers (67,68, 69, 71, 72) were studied in reaction mixtures containing some cofactors. However, one group (155) has attempted an evaluation of the cofactors. A probable reason for their failure to do so may stem from the weak ability of their preparations to evolve ethylene. The one exception was a paper by Chandra, Spencer and Meheriuk (155) that dealt with the effects of the components of a reaction mixture.

Factors evaluated by the author were those involved in substrate oxidations, decarboxylations, transaminations, and enzyme integrity. Problems that may be encountered in a study of this nature include the following:

- i) Is the concentration of the cofactor optimum for activity?
- ii) Do temperature, pH and ionic strength affect the actions of cofactors?
- iii) Does the cofactor function more efficiently in the presence of other cofactors?

A thorough appraisal of these problems could not be attempted but consideration was, nevertheless, given to them. First, some cofactors were studied at two concentrations to determine whether saturation had occurred or not. Secondly, temperature and pH were maintained at constant values of 25° C and 7.2 units, respectively.

Finally, in some experiments the effects of two cofactors added simultaneously were evaluated.

Resolution of the problems mentioned above is more meaningful with a purified enzyme powder than it is with a particulate fraction. The latter is a heterogeneous system of enzymes that fluctuates with the physiological state of the fruit and consequently, each preparation would be slightly different from the next insofar as composition and activity are concerned. An optimum concentration value determined in one run need not necessarily agree with subsequent determinations. Furthermore, the reactions available for cofactors in a particulate system are numerous but a purified enzyme preparation would be limited in enzymic reactions. A more comprehensive understanding of the function of the cofactor would, therefore, be gained from the purified enzyme preparation.

Pertinent to saturation limits of any cofactor is the effect of the endogenous source of the cofactor. It may be sufficient for most enzymic purposes and addition of an exogenous source would probably have no further effect. One might, therefore, be misled to interpret the effect of the added cofactor. Alternatively, addition of an exogenous source may be inhibitory to enzyme activity. The supposition is partly confirmed in a paper by Younathan (156) who found exogenous nicotinamide dinucleotide (NAD) to inhibit adenosine triphosphatase activity.

Nevertheless, the results obtained with the tomato particulate fraction will enable a better understanding of ethylene biosynthesis and can be of valuable assistance to people working with purified enzyme preparations.

Results and Discussion:

The results of the effects of cofactors on ethylene production are given in Table II (pages 65 -69).

a) Bovine Serum Album (1 mg/ml) : Runs 1, 2.

Ethylene production decreased slightly in the presence of bovine serum albumin.

The observation that no increase in ethylene production occurred when protein was added to the particulate suspension implies that its protection of the ethylene synthesizing enzymes is not required. Enzyme preparations are often exempted from denaturation and inactivation by the addition of bovine serum albumin. One function suggested for the protein in its protective capacity is that of binding cations deleterious to enzymic activity. Why a decrease in ethylene evolution should have occurred with the protein is not known. One suggestion that comes to mind is an interference of enzyme-substrate proximity.

b) Coenzyme A (7×10^{-5} M or 0.1 mg/ml) : Runs 3, 4.

A decrease in the yield of the gas was observed with catalytic amounts of coenzyme A (CoA), namely, 0.1 mg per ml of suspension.

The decrease in ethylene production on addition of CoA could have resulted from the mediation of a reaction detrimental to the synthesis of ethylene or it could have arisen from the formation of the acyl derivative of an intermediate in the pathway, the acylated derivative being preferentially metabolized to some other product. For instance, acylation of propionate to propionyl-CoA may result in carboxylation to form succinyl-CoA rather than dehydrogenation to acrylate and subsequent decarboxy-

lation to ethylene. No stimulation in ethylene evolution was observed by Abeles and Rubenstein (73) with an enzyme preparation that had CoA added to it.

c) Flavin Adenine Dinucleotide ($1.2 \times 10^{-4}M$ or 0.1 mg/ml)

Runs 5, 6.

Although the initial production of ethylene was elevated by the cofactor (FAD), that from the aged resonicated particles was reduced.

Since the decrease in production occurred during the 22 - 24 hr. period there is the possibility that sonication at the end of the 3 - 22 hr. period may have disrupted an enzyme complex containing FAD, and subsequent reorganization was either poorly achieved or was such that its activity was seriously depressed. The postulation of such a complex becomes more meaningful in view of the sustained ethylene production for the overnight or 3 - 22 hr. period, especially noticeable with the addition of flavin mononucleotide to the suspension.

d) Flavin Mononucleotide ($2 \times 10^{-4}M$ or 0.1 mg/ml):Runs 7, 8.

The presence of flavin mononucleotide (FMN) also resulted in a reduced yield of ethylene from the aged, resonicated particles. However, a significant increase in production of the volatile for the 3 - 22 hr. period was recorded.

The comments with respect to FAD are entirely applicable to the discussion of the results obtained with FMN. The point that some enzyme systems may be reconstituted should be stressed because of the significant overnight production of ethylene in

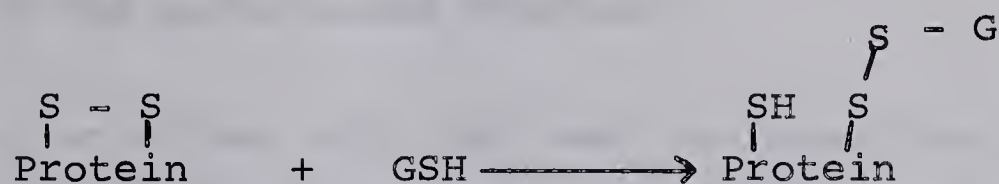
presence of FMN. In fact, experiments conducted with FMN and β -alanine showed a similar trend for the same period.

Abeles and Rubenstein (73) reported that FMN and Fe^{++} in the presence of substrate isolated from the pea homogenate evolved ethylene at a rate greatly exceeding that possible with the enzyme and substrate. However, since no details were given little can be said about the relevance of this observation to the tomato particulate fraction.

e) Glutathione ($1 \times 10^{-2}\text{M}$) : Runs 9, 10.

A pronounced decrease in ethylene production by the particles was observed when reduced glutathione (GSH) was added to the suspension.

The marked inhibitory effect of GSH may have resulted from the reduction of essential disulfide linkages. The overall reaction would be,



Reduction of disulfide bonds in wheat proteins by glutathione has been reported by Frater and Hird (157). The concept that disulfides may be important to ethylene biosynthesis stems from the inhibitory effect of cyanide (see Ch. 5), which also can reduce these linkages.

A second cause for the inhibition of ethylene production on addition of glutathione may have been the maintenance of a reduced state in the suspension, and moieties like α -lipoic acid that must undergo oxidation as well as reduction may not have been permitted to do so.

f) α -Lipoic acid (1×10^{-3} M):Runs 11, 12.

Slight increases in total ethylene production were measured from the suspensions containing α -lipoic acid.

The cofactor is normally associated with oxidative decarboxylation and enzymes like pyruvic and α -ketoglutaric dehydrogenase contain it. However, the slight increase observed with α -lipoic acid is insufficient to identify the presence of an oxidative decarboxylation step in the biosynthesis of ethylene. Since α -lipoic acid can exist as lipoic dehydrogenase not bound to enzymes concerned with oxidative decarboxylation (158) it is conceivable that the weak stimulation caused by the cofactor is that of a reaction other than oxidative decarboxylation.

g) Nicotinamide Adenine Dinucleotide (NAD):Runs 13, 14, 15, 16.

NAD at 1.5×10^{-4} M and 3.0×10^{-4} M had no influence on ethylene evolution by the particulate fraction.

The lack of an effect with NAD need not mean that a requirement for the cofactor does not exist. The possibility that sufficient NAD may reside in the suspension is not to be overlooked. Mitochondria, in general, contain more NAD than NADP (159) and sonication of mitochondria results in the liberation of these nucleotides (128, 160, 161, 162). If the endogenous source of NAD in the tomato particulate fraction were ample then an exogenous source of the cofactor would not, in all likelihood, influence the production of ethylene. Also pertinent to the discussion on NAD is the observation that citric acid cycle oxidations are disrupted in fragmented mitochondria (128, 163). (Citric acid

cycle oxidations are implicit in the pathways proposed by Burg and Burg (103) and Wang et al (53, 54).)

h) Nicotinamide Adenine Dinucleotide Phosphate:Runs 17, 18, 19, 20.

Two levels of NADP were used namely, $1.3 \times 10^{-4}M$ and $4.0 \times 10^{-4}M$ and increases of approximately 10% in the total yield of ethylene were realized from both levels.

Confirmation of an NADP linked dehydrogenase cannot be made on the evidence of the weak stimulation caused by NADP. Perhaps greater stimulation might have occurred if an endogenous source was not present in the particulate suspension. Therefore, it may not be presumptuous to consider the possible involvement of an NADP linked dehydrogenase in ethylene biosynthesis. Use of the cofactor in β -alanine studies disclosed a significant stimulatory effect on the metabolism of the amino acid. No effect with NADP was observed by Abeles and Rubenstein (73) on their pea enzyme preparation. Incidentally, the higher level of NADP was used in subsequent experiments to ensure an adequate supply of the cofactor since preparations would vary in their endogenous content of it.

i) Nicotinamide Adenine Dinucleotide and Coenzyme A:Runs 21, 22.

The addition of NAD ($3 \times 10^{-4}M$) and CoA ($7 \times 10^{-5}M$) together resulted in a slight increase in ethylene production for the 22 - 24 hr. collection period.

The synergistic effect observed with NAD and CoA is consistent with the idea that a complex, or a series, of enzymes may be of

importance to ethylene biogenesis. Results obtained with the flavin coenzymes, FAD and FMN, also support this contention primarily from the significant overnight production of ethylene measured when either was added to the particulate fraction. A synergistic effect was described by Lieberman and Biale (164) in the oxidation of α -keto acids by sweet pea mitochondria. They noted that CoA was effective only in the presence of NAD and TPP. This reference was mentioned because an α -keto acid, namely, α -ketobutyrate, has been implicated as an intermediate in ethylene biosynthesis (see Chp. 3). Considerable work with numerous combinations of cofactors should provide a complete description of any such complex.

j) Nicotinamide Adenine Dinucleotide Phosphate and Coenzyme A
: Runs 23, 24.

Production of ethylene was stimulated by 10% in the presence NADP ($4 \times 10^{-4}M$) and CoA ($7 \times 10^{-5}M$).

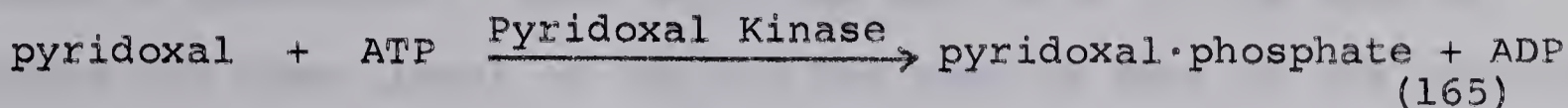
It would appear that a synergistic effect between CoA and NADP did not take place because the stimulation with NADP was the same as that observed with both cofactors together. Some effect, nevertheless, must have been exerted on CoA by NADP because the former as a cofactor reduced the production of ethylene and yet in the presence of NADP no decrease was evident. It is possible that NAD and NADP are preventing the CoA derivative of an intermediate from being siphoned off to another metabolic fate other than ethylene and thus coerce it towards ethylene formation. To cite an example, propionyl-CoA may undergo

dehydrogenation to acrylyl·CoA by NAD or NADP containing enzymes and then proceed to ethylene, whereas in the absence of NAD or NADP propionyl·CoA may be carboxylated to form methylmalonyl·CoA.

k) Pyridoxal (hydrochloride) ($5 \times 10^{-4}M$) : Runs 25, 26.

A slight increase of 10% in total ethylene production was caused by pyridoxal hydrochloride.

Pyridoxal enzymes are involved in a number of amino acid decarboxylations and transaminations. The slight increase observed with the cofactor may confirm the presence of transamination, a process already suggested by inhibitor studies (see Chp. 5). Since pyridoxal phosphate is the prosthetic group of the above enzymes pyridoxal hydrochloride is probably converted to this derivative.



l) Pyridoxal Phosphate ($5 \times 10^{-4}M$) : Runs 27, 28, 29, 30.

The effects of pyridoxal phosphate were somewhat inconsistent for ethylene production during the 22 - 24 hr. period. However, total production of the volatile decreased slightly in the presence of the cofactor. Addition of $10^{-4}M$ Fe^{+++} (as $Fe_2(SO_4)_3$) was without effect.

The inconsistency given by pyridoxal phosphate could be a result of varying amounts of an endogenous supply of the cofactor. It is recognized that enzymes containing pyridoxal phosphate as a prosthetic group bind the cofactor firmly and little dissociation occurs during the isolation of the particulate fraction. The inconsistency may be a reflection of the dissociation. A lack

of effect with Fe^{+++} may suggest that the transaminase involved in ethylene production does not require a trivalent cation for activity, or that sufficient is already present.

m) Thiamine Pyrophosphate : Runs 31, 32, 33, 34, 35, 36, 37, 38.

Thiamine pyrophosphate (TPP) at a level of $2 \times 10^{-3}\text{M}$ (1 mg/ml) increased ethylene production slightly more than 20%. Addition of $1 \times 10^{-3}\text{M}$ Mg^{++} (as MgSO_4) resulted in a further elevation of the yield of ethylene. A lower concentration of TPP, namely, $2 \times 10^{-4}\text{M}$ (0.1 mg/ml) gave only a slight increase in ethylene evolution for the 22 - 24 hr. collection period. Addition of $1 \times 10^{-3}\text{M}$ Mg^{++} had little effect on ethylene production by the particulate suspension. Total ethylene evolution in the studies employing the lower level of TPP was the same as that of the controls

The stimulation observed with TPP and Mg^{++} is highly indicative of oxidative or non-oxidative decarboxylation. Transketolase activity, which is stimulated by these cofactors, is not suspected because the enzyme is located in the soluble phase of the cell. If decarboxylation of acrylate can be assumed as the final step to the formation of ethylene then the stimulatory effects of TPP and Mg^{++} support this non-oxidative decarboxylation. However, if acrylyl·CoA is the primary intermediate then oxidative decarboxylation is required and the weak stimulatory effects of NADP and α -lipoic acid coupled with the effects of TPP and Mg^{++} support this contention. Decarboxylation is also implied in the conversion of α -ketobutyrate to propionate (see Chp. 3).

The greater stimulatory effect of TPP at the higher concentration may be attributed to two reasons. First, it may be ample to supply several enzymes competing for it. Secondly, should TPP be catabolized readily then the higher level of TPP could compensate for any loss, a situation not possible with the lower concentration of the cofactor. Metabolism of TPP in the particulate suspension is a speculative thought.

In summary the results with TPP and Mg^{++} point to decarboxylation but whether it is oxidative or non-oxidative cannot be definitely stated. The results with α -lipoic acid are inconclusive to support oxidative decarboxylation and may perchance indicate a reaction other than the latter process. The inhibitory effect of reduced glutathione supports the importance of disulfide linkages in enzymes to ethylene production. The slight increase in ethylene evolution caused by NADP may mean that an NADP-linked dehydrogenase is involved in the biosynthesis of the volatile. The significant increase in the overnight (3 - 22 hr.) yield of ethylene in the presence of FMN suggests reconstitution of a complex or enzyme sequence as does the synergistic effect between CoA and NAD or NADP. Finally, the inconsistent effect with pyridoxal phosphate does not enable positive identification of transamination.

Table II. Effect of Several Cofactors on Ethylene Production
by a Particulate Fraction from Tomatoes

C ₂ H ₄ (μul)													
Bovine Serum Albumin (1mg/ml)				Coenzyme A (7 x 10 ⁻⁵ M)				Flavin Adenine Dinucleotide (1.2 x 10 ⁻⁴ M)				Flavin Mononucleotide (2 x 10 ⁻⁴ M)	
Run No.	1			3			5			7			
Period	A	B	%	A	B	%	A	B	%	A	B	%	
0 - 3 h	194	208	+ 7	242	144	-40	180	264	+47	180	177	- 2	
3 - 22 h	45	17		43	23		N	73		N	248		
22 - 24 h	2430	2145	-12	1423	1227	-14	1315	1095	-17	1315	1066	-19	
Total	2669	2340	-12	1708	1393	-18	1495	1431	- 4	1495	1491		
Nitrogen (mg)	8.41	8.14		8.79	8.79		8.05	8.31		8.05	8.31		
Run No.	2			4			6			8			
0 - 3 h	313	231	-26	276	270	- 2	118	182	+54	118	101	-14	
3 - 22 h	59	47		51	73		59	90		59	119		
22 - 24 h	945	824	-13	2469	2013	-18	1690	1220	-28	1690	1083	-36	
Total	1317	1112	-16	2796	2359	-16	1867	1492	-20	1867	1403	-25	
Nitrogen (mg)	5.87	5.69		8.19	8.02		8.75	7.88		8.75	8.49		

Table II. (cont'd)

C ₂ H ₄ (m μ L)											
Glutathione (Reduced) (1 x 10 ⁻² M)				α -Lipoic Acid (1 x 10 ⁻³ M)				Nicotinamide Adenine Dinucleotide (1.5 x 10 ⁻⁴ M)			
								Nicotinamide Adenine Dinucleotide (3.0 x 10 ⁻⁴ M)			
Run No.	9			11			13			15	
Period	A	B	%	A	B	%	A	B	%	A	B %
0 - 3 h	220	174	-21	411	621	+51	225	244	+ 8	315	293 - 7
3 - 22 h	N	113		N	N		N	14		34	116
22 - 24 h	3308	1671	-49	3650	3742	+ 3	749	698	- 7	620	534 -14
Total	3538	1958	-45	4061	4363	+ 7	974	956	- 2	969	943 - 3
Nitrogen (mg)	4.55	4.55		12.11	11.98		7.79	7.88		6.56	6.77
Run No.	10			12			14			16	
0 - 3 h	220	287	+30	358	329	- 8	118	137	+16	236	227 - 4
3 - 22 h	N	N		77	107		59	N		N	N
22 - 24 h	3308	1384	-58	956	1057	+11	1690	1696		488	489 0
Total	3538	1671	-53	1391	1493	+ 7	1867	1833	- 2	724	716 - 1
Nitrogen (mg)	4.55	4.76		5.86	5.51		8.75	8.58		7.00	7.00

Table II. (cont'd)

C ₂ H ₄ (m μ l)											
Nicotinamide Adenine Dinucleotide Phosphate (1.3 x 10 ⁻⁴ M)				Nicotinamide Adenine Dinucleotide Phosphate (4.0 x 10 ⁻⁴ M)				NAD (3 x 10 ⁻⁴ M) CoA (7 x 10 ⁻⁵ M)			
								NADP (4 x 10 ⁻⁴ M) CoA (7 x 10 ⁻⁵ M)			
Run No.	17			19			21			23	
Period	A	B	%	A	B	%	A	B	%	A	B %
0 - 3 h	282	343	+22	123	124		236	231	- 2	250	153 -39
3 - 22 h	70	30		39	51		N	N		N	65
22 - 24 h	1100	1283	+17	911	956	+ 5	488	540	+11	1690	1710 + 1
Total	1452	1656	+14	1073	1131	+ 5	724	771	+ 6	1940	1928 - 1
Nitrogen (mg)	6.04	6.16		10.33	10.33		7.00	7.89		8.40	8.49
Run No.	18			20			22			24	
0 - 3 h	199	203	+ 2	152	264	+74	219	225	+ 3	191	126 -34
3 - 22 h	8	N		56	99		68	N		17	73
22 - 24 h	880	1027	+17	1138	1325	+16	962	1022	+ 6	975	1026 + 5
Total	1087	1230	+13	1346	1688	+25	1249	1247		1183	1225 + 4
Nitrogen (mg)	6.83	6.86		8.14	8.23		7.44	8.23		7.61	7.93

Table II. (cont'd)

C ₂ H ₄ (m μ l)												
Pyridoxal Hydrochloride (5 x 10 ⁻⁴ M)				Pyridoxal Phosphate (5 x 10 ⁻⁴ M)				Pyridoxal Phosphate* (1 x 10 ⁻⁴ M) Fe ⁺⁺ (1 x 10 ⁻⁴ M)				Thiamine Pyrophosphate (2 x 10 ⁻³ M)
Run No.	25			27			29			31		
Period	A	B	%	A	B	%	A	B	%	A	B	%
0 - 3 h	152	253	+66	306	225	-26	366	360	- 2	422	355	-16
3 - 22 h	56	23		39	N		39	N		N	23	
22 - 24 h	1138	1188	+ 4	647	715	+11	1592	1569	- 1	540	704	+30
Total	1346	1464	+ 9	992	940	- 5	1997	1929	- 3	962	1182	+23
Nitrogen (mg)	8.14	8.68		6.74	7.18		9.36	9.63		11.99	10.67	
Run No.	26			28			30			32		
0 - 3 h	230	253	+10	307	244	-21	244	281	+15	452	618	+37
3 - 22 h	51	N		73	39		39	90		200	230	
22 - 24 h	587	691	+18	1575	1417	-10	1417	1450	+ 2	805	973	+21
Total	868	944	+ 9	1955	1700	-13	1700	1821	+ 7	1457	1821	+25
Nitrogen (mg)	7.00	6.74		7.35	7.37		7.37	7.37		4.54	4.04	

* control contains pyridoxal phosphate

Table II. (cont'd)

C ₂ H ₄ (mμl)									
TPP** ₊₊ (2 x 10 ⁻³ M) Mg (1 x 10 ⁻³ M)				Thiamine Pyrophosphate (2 x 10 ⁻⁴ M)				TPP** ₊₊ (2 x 10 ⁻⁴ M) Mg (1 x 10 ⁻³ M)	
Run No.		33		35		37			
Period		A	B	%	A	B	%	A	%
0 - 3 h		253	191	-24	306	242	-21	84	+54
3 - 22 h		28	34		39	14		N	N
22 - 24 h		1395	1548	+11	647	691	+ 7	925	+ 1
Total		1676	1773	+ 6	992	947	- 5	1009	+ 7
Nitrogen (mg)		9.38	9.38		6.74	7.00		5.81	6.17
Run No.		34		36		38			
0 - 3 h		161	101	-37	306	233	-23	169	-17
3 - 22 h		N	N		39	23		122	73
22 - 24 h		1217	1647	+35	647	723	+12	560	+14
Total		1378	1748	+27	992	979	- 1	851	855
Nitrogen (mg)		6.76	6.58		6.74	6.48		5.69	5.60

Basic Reaction Mixture
0.5M sucrose
0.125M KH₂PO₄
pH 7.2
1.9 x 10⁻⁵M ATP added
to each flask.
Particles were soni-
cated for 4 min. at
1.2 amp at beginning
of the collection
periods 0-3 hr. and
22-24 hr., respectively

A = control containing
particles suspended
in basic reaction
mixture but without
added cofactor

B = reaction vessel con-
taining the same as
A but with added
cofactor

% = percent increase or
decrease in ethylene
production

N = non detectable

T = trace amount

** control contains TPP
*** control contains TPP

5. Effects of Various Inhibitors on Ethylene Production

Considerable information concerning the degradation of an organic compound in biological systems can be acquired from the judicious use of respiratory inhibitors. To cite an example, inhibition in the metabolism of an organic acid by iodoacetate suggests that an enzyme containing sulfhydryl groups essential to its activity is directly involved in the metabolism of the acid. However, with the complex enzyme system that would be found in a particulate fraction the identification of individual enzymes by use of inhibitors is not a simple task. This is especially true when the number of inhibitors employed in a study is limited to one or two. But use of several inhibitors and careful interpretation of the results can identify a pathway or an enzyme or do both. The administration of two inhibitors together is a procedure often followed because inhibition patterns not observed with either inhibitor by itself become evident.

Inhibition studies like the one conducted by the author invite the consideration of some problems. One problem that is difficult to diagnose is whether the inhibition of an enzymatically catalyzed process causes the normal state of metabolism to revert to a secondary state of metabolism that is not indicative of an in vivo state. This is especially true when high concentrations of an inhibitor are used. Inactivation of several enzymes by an inhibitor could create a new biochemical state unrelated to in vivo conditions and interpretation of results from such studies may be completely misleading.

It is not always possible to know whether an inhibitor is acting solely on an enzyme or reacting with some non-enzymic constituent. Occurrence of the latter action would reduce the effective concentration of the inhibitor. Another consequence of the reaction might be : non-enzymic inhibition that would be interpreted as enzyme in nature.

Since many inhibitors are weak bases and acids their activity depends on the pH of the medium in which they are to function. The pH of the medium will determine whether the inhibitor will be dissociated or not, a feature often vital to the inhibitory action.

Inhibition of an enzymic reaction may result in the formation of an intermediate that is inhibitory to an enzymic process in the same pathway or in some other pathway. In fact, an entire sequence of feedback inhibition involving several pathways may emerge. The formation of an excess amount of 'an intermediate may activate a pathway not usually accessible to the metabolism of that compound.

Metabolism of the inhibitor is a problem more often encountered with inorganic inhibitors than with organic inhibitors. Whenever metabolism of an inhibitor is suspected, or known to occur, interpretation of the results should be assessed in terms of this fact.

Penetration of inhibitors into tissue is another problem. Some inhibitors readily penetrate the membranes of cells and cellular particles but penetration of others is pH-dependent. In the fragmented particulate fraction from tomatoes penetrations of inhibitors presents no difficulty.

Lack of specificity is a major problem encountered with inhibitors. Inhibition of one enzyme by an inhibitor acting within a multiple enzyme complex is not always possible. However, advantage is taken of the fact that some enzymes are more sensitive to a particular inhibitor than are other enzymes and preferential inhibition may be achieved with low levels of an inhibitor.

The current study of inhibitors was used to evaluate the contributions of major metabolic pathways to the production of ethylene. Determination of the concentrations for maximum inhibition was not attempted because numerous enzymes would be affected and identification of pathways would be impossible.

Invaluable information about the mode of action of inhibitors was obtained from the treatises, "Metabolic Inhibitors", edited by R.M. Hochster and J.H. Quastel (166, 167). The two volumes comprise a comprehensive treatment of inhibitors, their mode of action, their experimental conditions of study and interpretation of their results. A review on inhibition in plants by James (168) was also consulted.

Results and Discussion

The results obtained from the effects of various inhibitors on ethylene production by a particulate fraction from ripening tomatoes are presented in Table III (page 91).

a) Arsenite (sodium salt) : Runs 1, 2, 3, 4, 5, 6.

Arsenite is often used as an inhibitor of oxidative decarboxylation of α -keto acids but many other enzymes other than those involved in the foregoing process are affected by the reagent (167).

At a level of 10^{-4} M, arsenite had no effect on ethylene production but at 10^{-3} M a significant decrease in the production of the volatile was observed. When the concentration of arsenite was raised to 10^{-1} M, production for the 22 - 24 hr. collection period was inhibited by 85% or more. In nearly all the runs the initial production was higher in the presence of arsenite.

The higher concentration of arsenite may have completely inhibited an enzyme responsible for ethylene biosynthesis or it may have inactivated several enzymes indirectly associated in the biosynthesis of the gas. However, too much confidence in the interpretation of the results with the high level of arsenite cannot be assumed for reasons specified in the introduction to this chapter.

Inhibition of oxidative phosphorylation (169, 170, 171) may be a cause for the reduction of the yield of ethylene by arsenite. The process is known to occur in fragmented mitochondria (172, 173). That energy in the form of ATP is stimulatory to ethylene production was demonstrated by Chandra and Spencer (97, 155).

An inhibitory effect of arsenite on α -ketoglutaric dehydrogenase (174) must be regarded with caution. Although α -ketoglutarate did not stimulate ethylene production when added to the particulate fraction, this does not preclude a role for the enzyme as an adequate endogenous supply of the organic may be present. Inhibition of α -ketoglutaric dehydrogenase activity would reduce the amount of NADH oxidized by the cytochromes and thus, the supply of

ATP would

decrease. The inhibition of succinate formation is not considered as a significant event of α -ketoglutarate dehydrogenase inactivation since malonate at high levels was not inhibitory to ethylene production (see Section k).

Because of its affinity for thiol groups arsenite inhibits transaminase activity, fatty acid metabolism and glucose metabolism as well as other reactions (167) but whether all of these are involved in ethylene biosynthesis cannot be stated in view of the limited knowledge known about the ethylene producing system.

b) Aminooxyacetic Acid (hemihydrochloride): Runs 7,8

Mild inhibition of the evolution of ethylene was caused by aminooxyacetic acid ($\text{NH}_2\text{-O-CH}_2\text{-COOH}$) at 10^{-3}M .

The reagent was first reported by Wallach (175) who found it to inhibit a γ -aminobutyric acid transaminase. Hopper and Segal (149, 176) reported inhibition of a glutamic-alanine transaminase with the reagent. They also stated that a pH range of 7.5 - 8.1 resulted in maximum inhibition.

Transamination is necessary for the entry of glutamate and aspartate into pathways (1) and (3) as described in the introduction of Chapter 3. It is also necessary for the conversion of β -alanine to malonic semialdehyde in the pathway proposed by Thompson and Spencer (74). The author has proposed a transamination reaction in the production of aspartic acid from glutamic acid. Aspartic acid is a precursor to homoserine, an amino acid that was postulated to be relevant to ethylene production (see discussion Chapter 3).

c) Azide (sodium salt) : Runs 9, 10.

Azide (N_3^-) was found to decrease total ethylene production by about 35% when used at a concentration of 10^{-3} M. The mode of action of azide is that of chelation with metal ions and thus metallo-proteins are inhibited by the agent. It reacts with cytochrome oxidase and cytochrome C in the electron transport system. Stannard and Horecker (177) reported that azide reacted with cytochrome C in the acidic form as HN_3 . Extensive inhibition of the cytochrome would not be expected in the suspension of pH 7.2 because azide would be almost completely in the salt form since its pKa value is 4.7.

Gibson (52) observed complete cessation of ethylene production by Penicillium digitatum when 10^{-2} M azide was added. Abeles and Rubenstein (73) reported a 50% inhibition of ethylene production by a pea enzyme preparation subjected to 10^{-3} M azide.

d) p-Chloromercuribenzoate (sodium salt) : Runs 11, 12, 13, 14, 15, 16.

Since the solubility of the inhibitor is limited in water, it was initially dissolved in a small quantity of weak alkali (0.1 N NaOH) and then transferred quantitatively to the reaction flask. The two concentrations of p-chloromercuribenzoate (p-CMB) used, 10^{-4} M and 10^{-3} M, were both inhibitory to ethylene production. The extent of inhibition was about 25% and 50%, respectively. Reversal of p-CMB inhibition at 10^{-4} M was obtained by addition of 10^{-2} M glutathione. The thiol reagent was added one hour after initial incubation of the inhibitor with the particulate suspension.

Inhibition of enzymes by p-CMB results from the formation of a mercaptide between a sulfhydryl group on the enzyme, and the inhibitor.



(the halogen was replaced by a hydroxyl ion when p-CMB was dissolved in the weak alkali)

The results from p-CMB inhibition indicate the necessity of essential sulfhydryl groups to the biogenesis of ethylene.

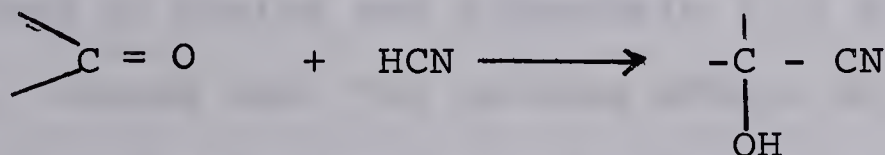
e) Cyanide (sodium salt) : Runs 17, 18, 19, 20, 21, 22.

Use of 1 or 2×10^{-4} M cyanide resulted in no inhibition of ethylene production but cyanide at 10^{-2} reduced the yield of ethylene for the 22 - 24 hr. collection period by 35%. Initial production of the volatile was higher in the presence of cyanide, a result also observed with arsenite.

Cyanide can repress enzyme activity in three ways. The first mode of action is the reduction of disulfide linkages in proteins.



Second, is the reaction with carbonyl groups to form cyanohydrins.



The third mode of action is chelation with the metal moiety of metallo-proteins.

Apparently, cyanide reacts with protein carbonyl groups as the acid, HCN, and only when a high concentration of the inhibitor

is present (167). Since the pK_a of cyanide is 9.14 a substantial quantity of HCN should be present in the particulate suspension of pH 7.2. The reaction is a reversible one but that of the reduction of disulfide linkages is not.

Cytochrome oxidase and cytochrome C are both sensitive to cyanide. Moreover, a high concentration of the inhibitor inactivates the succinoxidase system by reduction of a disulfide bond in succinic dehydrogenase. Non-enzymic constituents also react with cyanide and included are α -keto acids, aldehydes and pyridoxal phosphate. Transaminases are inhibited because of the reaction between cyanide and their prosthetic group, pyridoxal phosphate.

Gibson (52) observed progressive inhibition of ethylene evolution by Penicillium digitatum as the cyanide concentration steadily increased to 5×10^{-4} M. Hansen (32) observed no effect on ethylene production by pears when low levels of HCN were used. High levels of the reagent reduced the yield of ethylene, but they also induced severe tissue damage. Lieberman and Mapson (76) reported inhibition of ethylene production by apple plugs treated with 10^{-3} M cyanide. A 50% reduction in the yield of ethylene was observed by Abeles and Rubenstein (73) with a pea preparation. Burg (181) stated that "no obvious effect on ethylene production" by apple discs was evident when subjected to 10^{-2} M cyanide. A drastic reduction in cyanide concentration because of its reaction with non-enzymic constituents not essential to ethylene production may have accounted for the lack of effect by cyanide in this tissue.

However, a penetration problem must not be overlooked as a cause.

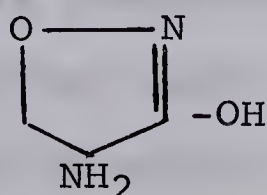
In our preparations inhibition of a metallo-enzyme by cyanide is a reasonable assumption in view of the corroborating results of azide. Whether the enzyme is cytochrome oxidase, some other enzyme or cytochrome C cannot be stated. Reaction with carbonyl groups is relevant to transamination, a process already suggested to be involved in ethylene biosynthesis (see Chapter 3), and also to other enzymic processes as well as to non-enzymic constituents. The reduction of disulfide linkages in proteins is also a possibility especially in view of the inhibition observed with glutathione (see Chapter 4). All three modes of action for cyanide are, therefore, candidates for the basis or bases of inhibition of ethylene production by the tomato particulates system.

Caution, however, must be exerted in interpretation of results from cyanide inhibition, since cyanide has been reported to be metabolized and assimilated by some plants (178, 179, 180).

f) D-Cycloserine : Runs 23, 24

Appreciable inhibition of ethylene production occurred in the presence of cycloserine. A comprehensive review on cycloserine inhibition of transaminases has been prepared by Braunstein, Azarkh and Tin-Sen (182). The mode of action proposed by the above authors for cycloserine is by competition with pyridoxal phosphate and by binding to the active site of the enzyme.

Structurally, cycloserine is

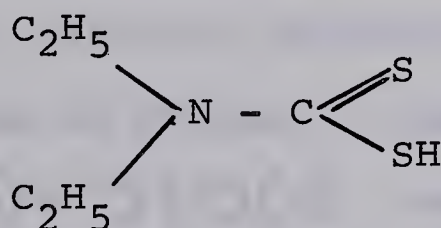


The stronger inhibition of ethylene production by cycloserine over that of aminooxyacetic acid may indicate a transaminase more sensitive to cycloserine or it may indicate inhibition of another transaminase.

g) Diethyldithiocarbamate (sodium salt) : Runs 25, 26, 27, 28.

Ethylene production from the aged, resonicated suspension was reduced significantly by the two concentrations of diethyldithiocarbamate (DIECA) used.

DIECA inhibits enzymes primarily by its chelation with metal ions and thus, acts similarly to azide and cyanide. It can also inhibit succinic dehydrogenase by oxidizing the thiol groups present in the enzyme (167). Oxidation is caused by tetraethyldithiocarbamyl sulfide, a compound formed when DIECA is oxidized by the cytochrome system. The structural formula for DIECA is



Lieberman and co-workers (72, 76, 78) observed inhibition of ethylene production by DIECA with apple cytoplasmic particles and apple plugs. A copper enzyme was suggested as the enzyme inactivated.

A metallo-protein is undoubtedly inhibited by DIECA but to suggest that it contain copper is premature. It would be interesting to speculate whether cyanide, azide, and DIECA are acting on the same enzyme. How much of an effect DIECA exerts on

succinic dehydrogenase will depend upon the extent of DIECA oxidation by the cytochrome system.

h) Fluoride (sodium salt) : Runs 29, 30, 31, 32.

At 10^{-3} M fluoride did not inhibit ethylene production but when elevated to 10^{-1} M it decreased production of the olefin for the 22 - 24 hr. period by about one third.

The classical explanation for the effect of fluoride is its inhibition of enolase activity in the glycolytic pathway. In the presence of phosphate an undissociable $[Mg][P_i][F^-]^2$ complex is formed that inactivates the enzyme. However, other enzymes found to be sensitive to fluoride include phosphoglucomutase, phosphatases and phosphorylases but a different mechanism of inhibition is suggested for these enzymes, namely, the formation of a $[Mg][F^-][Enzyme]$ complex. Not all enzymes requiring Mg^{++} are inhibited by fluoride. Succinic dehydrogenase is strongly inhibited by fluoride if phosphate is present. Competitive inhibition between succinate and a $[Fe^{+++}][F^-][P_i]$ complex is suggested as the cause of inhibition. The observation by Forti (183) that adenosine triphosphatase activity (ATPase) is inhibited by fluoride raises the possibility of a complex formation since phosphate is needed to stimulate the enzyme. Assessment of fluoride inhibition is complicated by the fact that plants metabolize the halogen (184),

Burg and Thimann (63) noted that fluoride inhibition of ethylene production by apple plugs was partially overcome by addition of ATP. Interference of oxidative phosphorylation was

proposed by them as inhibitory effect of fluoride. Abeles and Rubenstein (78) stated that ethylene evolution was reduced by 50% when 10^{-3} M NaF was added to a pea enzyme preparation.

The decrease in ethylene production by the tomato particulate fraction is not due to the inhibition of the glycolytic enzymes mentioned because the particulate constituents of the cell are not generally considered to contain the complete roster of the enzymes of the glycolytic pathway. Inhibition of succinic dehydrogenase would interfere with oxidative phosphorylation and consequently, jeopardize the supply of ATP, a high energy compound previously shown to stimulate ethylene production by the tomato particulate fraction (97, 155). Fluoride may also be inhibitory to some other enzyme associated with ethylene production. However, since a high concentration of the inhibitor was required to demonstrate inhibition, caution must be exercised in assuming that inhibition was a result of the usual mechanism of fluoride, whereas, in effect, it might be based on an entirely different reason.

i) Hydroxylamine (hydrochloride) : Runs 33, 34.

Ethylene production from the aged, resonicated particles was reduced by 50% in the presence of 10^{-3} M hydroxylamine.

The inhibitor is regarded as a potent carbonyl trapping agent and thus, reacts with the carbonyl group to form an oxime:



Formation of an oxime may inactivate enzymes, or it may prevent the utilization of non-enzymic constituents.

Decarboxylases for amino acids, and transaminases, which contain pyridoxal phosphate as a prosthetic group, are strongly inhibited by hydroxylamine. Acyl derivatives, in general, react with the reagent to form the corresponding hydroxamate.

Since hydroxylamine can react with numerous enzymes and non-enzymic constituents it is practically impossible to identify the locus for the inhibitory effect. Hydroxylamine would inhibit transamination, which the inhibition studies with D-cycloserine and aminooxyacetic acid support as being involved in ethylene production. The production of propionate from homoserine via α -ketobutyrate also would be inhibited by hydroxylamine (see Chapter 3, Results and Discussion). As a result of the latter a potential precursor to ethylene would become inaccessible. Hydroxylamine inhibition of ethylene production by a pea enzyme preparation was reported by Abeles and Rubenstein (73).

j) Iodoacetamide : Runs 35, 36, 37, 38.

The two concentrations of iodoacetamide used, 10^{-4} M and 10^{-2} M, caused a substantial decrease in ethylene production.

Iodoacetamide reacts slowly with sulfhydryl groups on enzymes.



The reaction of iodoacetamide with thiol groups is slower than that of p-CMB and is usually irreversible.

Burg and Thimann (63) found that the inhibition of ethylene production by apple plugs in the presence of 10^{-2} M iodoacetamide was not reversed by addition of ATP. Ethylene evolution from

Penicillium digitatum decreased by 22% when Gibson (52) added 10^{-4} M iodoacetate to the fungus. Inhibition less than 30% was reported by Abeles and Rubenstein (73) with a pea enzyme preparation that contained 10^{-2} M iodoacetate. Iodoacetate acts similarly to iodoacetamide.

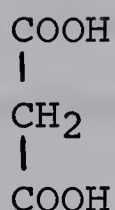
In comparing the inhibition from iodoacetamide and p-CMB one finds that at a concentration of 10^{-4} M iodoacetamide was more inhibitory to ethylene production. A 50% inhibition of ethylene production was observed with 10^{-2} M iodoacetamide and 10^{-3} M p-CMB. The result might be taken to mean that p-CMB reacts more readily with the thiol groups but since inhibition by 10^{-3} M iodoacetamide was not attempted it is presumptuous to make the allegation. The stronger inhibition of iodoacetamide at the lower level over that of p-CMB may indicate its ability to react with thiol groups inaccessible to p-CMB.

k) Malonic acid : Runs 39, 40, 41, 42.

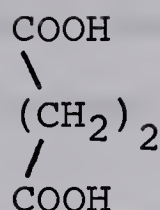
Malonic acid at 10^{-3} M and 10^{-2} M had no effect on ethylene production by the tomato particulate suspension.

Inhibition of the TCA cycle by malonate occurs at the succinic dehydrogenase reaction step. Since the affinity of the enzyme for malonate is three fold that for succinate competitive inhibition of the enzyme results. Oxaloacetate decarboxylase is another enzyme inhibited by malonate.

The structural formula of malonate closely resembles that of succinate.



malonic acid



succinic acid

Gibson (40) observed weak inhibition of ethylene production by Penicillium digitatum when 10^{-2} malonate was present.

Malonate inhibition in plants is difficult to achieve because the organic acid is readily metabolized (185, 186). Concentrations exceeding 10^{-2} M are usually required before inhibition takes place. The lack of inhibition observed in the current study may indicate no function for succinic dehydrogenase in ethylene biosynthesis, or that the concentration of malonic acid is effectively reduced through metabolism. However, the result of Gibson (52) would agree with the first contention.

1) Mercuric Sulfate : Runs 43, 44.

The effect of 10^{-2} M Hg^{++} on ethylene evolution by the aged resonicated suspension was pronounced and inhibition of production for the 22 - 24 hr. period amounted to 80%. Since some of the Hg^{++} formed a precipitate in the phosphate buffer it was not known what the effective concentration of the mercuric ions was in the suspension.

Heavy metals inactivate enzymes by reacting with their thiol groups. The mercaptide formed with Hg^{+2} would be,



Other groups on the protein that may react with Hg^{++} include phosphate, carboxyl and hydroxyl groups.

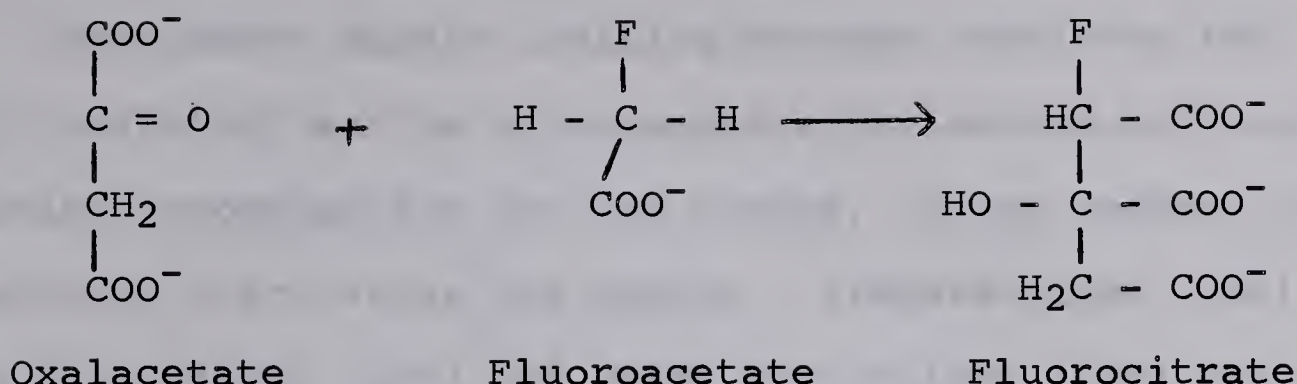
Abeles and Rubenstein (73) reported a 30% decrease in ethylene production by a pea enzyme preparation in the presence of 10^{-2}M Hg^{++} .

The rather potent inhibitory effect of Hg^{++} certainly indicates the need for thiol groups in ethylene production. However, there remained the possibility that Hg^{++} may have combined with some of the ethylene and thus, caused a reduction in the yield of the gas evolved. A preliminary experiment was done to verify or deny this possibility. To two sealed reaction vessels, both containing 25 ml of buffer (see Methods) and one containing the Hg^{++} , was added a known quantity of ethylene. The solutions were stirred gently for 22 hours, then flushed for 2 hours into mercuric perchlorate reagent and the regenerated ethylene analyzed by gas chromatography. Significant absorption of the injected ethylene by Hg^{++} was observed and consequently the effect of Hg^{++} must be, in part, attributed to absorption of some ethylene.

m) Monofluoroacetate (sodium salt) :Runs 45, 46, 47, 48.

Monofluoroacetate at 10^{-4}M and 10^{-3}M effected a weak inhibition in the production of ethylene by the aged, resonicated particles.

The inhibitory effect of monofluoroacetate is exerted on the TCA cycle (187) and results from the formation of fluorocitrate, which is not acted upon by aconitase.



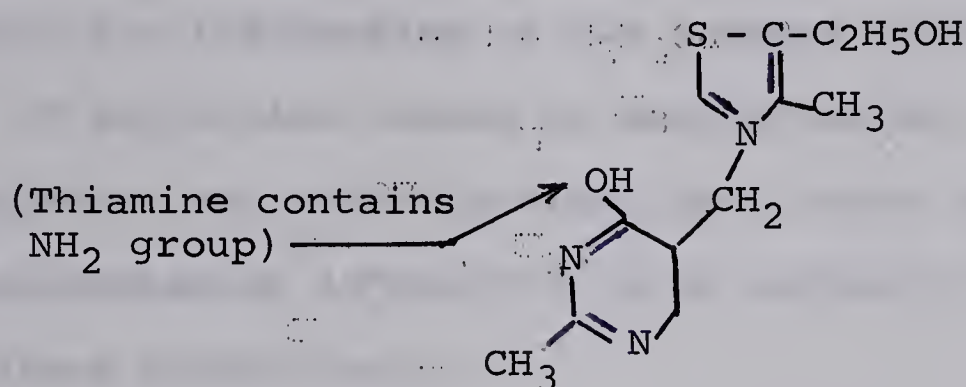
Burg and Thimann (181) observed progressive inhibition of ethylene production by apple discs while monofluoroacetate concentration steadily increased and at 10^{-1} M monofluoroacetate inhibited production by 70%. Gibson (52) reported a 56% inhibition of ethylene production by Penicillium digitatum subjected to 10^{-3} M monofluoroacetate.

The results from the tomato particulate fraction are somewhat at variance with those of Burg and Thimann and Gibson. However, in whole tissue the TCA cycle is tightly coupled with oxidative phosphorylation and blocking the cycle may interfere with ATP synthesis. Another consequence may be the accumulation of metabolic products inhibitory to ethylene production. Since Burg and Burg (103) and Wang et al (54) postulate a role for the TCA cycle in ethylene biosynthesis by whole tissue then monofluoroacetate inhibition of ethylene production is consistent. However, with a disrupted particulate fraction all TCA cycle oxidations are not coupled to each other (128, 163) and, therefore, monofluoroacetate may not have any effect on ethylene production.

n) Oxythiamine : Runs 49, 50.

Ethylene evolution was appreciably stimulated by oxythiamine at 10^{-3} M.

Oxythiamine mainly inhibits enzymes requiring TPP. It is only inhibitory as the pyrophosphate derivative and competes with thiamine pyrophosphate for the enzyme. As an enzymic cofactor it completely inactivates the enzyme. Transketolase (188), yeast acid phosphatase (189) and yeast carboxylase (190) are reported to be inhibited by oxythiamine. Structurally oxythiamine is

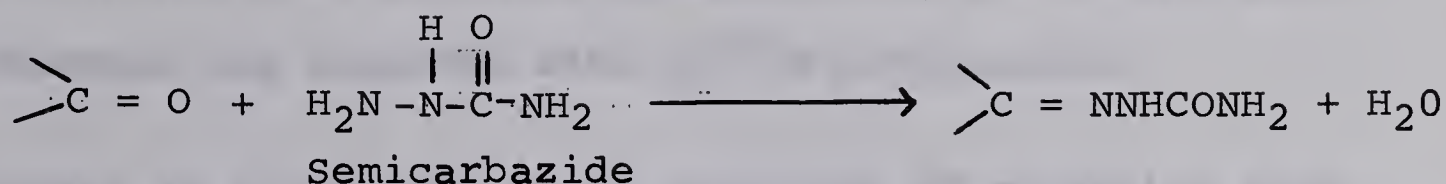


The stimulatory effect of oxythiamine is especially surprising in view of carboxylase activity proposed in Chapter 3. The enzyme is involved in the decarboxylation of α -ketobutyrate to propionate. Inhibition of transketolase activity, which is restricted to the soluble phase of the cell, cannot be the cause for the stimulation. An effect on phosphatase is possible although phosphoric esters have not been reported to be relevant to ethylene production. Perhaps the answer to the stimulation lies in determining whether the inhibitor acted as oxythiamine or as oxythiamine pyrophosphate.

o) Semicarbazide (hydrochloride) : Runs 51, 52.

Semicarbazide at 10^{-3} M inhibited total ethylene production by 20%. Initial production of ethylene was significantly lower in the presence of the inhibitor.

The reagent combines with carbonyl groups to form the corresponding semicarbazones:



Since the behavior of semicarbazide is similar to that of hydroxylamine the reader is referred to the discussion of the latter inhibitor for information on the enzymes inhibited. The lower degree of inhibition caused by semicarbazide compared to that of hydroxylamine may mean a greater reactivity for hydroxylamine, or that semicarbazide is reacting with carbonyl groups not important to ethylene production.

p) Silver Nitrate : Runs 53, 54.

Production of ethylene by the aged, resonicated particles decreased considerably in the presence of 10^{-2} M silver ions. The difficulty of precipitation in a phosphate buffer was also encountered with Ag^+ and consequently, the effective concentration of Ag^+ was unknown.

Inhibition of enzymes by Ag^+ results from mercaptide formation with their thiol groups.



The results with silver nitrate confirm the role of enzymes containing essential sulfhydryl groups in the biosynthesis of ethylene.

q) L-Thyroxine (sodium salt) : Runs 55, 56.

Weak inhibition of ethylene production by the tomato particulate fraction was observed with 10^{-3} M L-thyroxine.

Thyroxine is often used as an uncoupler of oxidative phosphorylation (166, 163) but it does not produce this effect with mitochondrial fragments (163). Therefore, the inhibition observed with thyroxine is not a result of an uncoupling of oxidative phosphorylation. A paper by Horvath (191) disclosed inhibition of an alanine glutamate transaminase by thyroxine. Since a transaminase has been implicated in ethylene biogenesis it is conceivable that thyroxine acts on the enzyme.

A summary of the results obtained with the inhibitors reveals several distinctive features. The role of sulfhydryl groups in ethylene biogenesis is corroborated by the inhibitory effects of Hg^{++} , Ag^+ , arsenite, p-CMB and iodoacetamide. The necessity of disulfide linkages is supported by cyanide and glutathione (see Chapter 4) inhibition. Inhibition by azide and cyanide may be primarily on the cytochrome system but other metallo-enzymes may be inactivated. The inhibitory effect of DIECA indicates the necessity of metal ions in ethylene biosynthesis. Carbonyl groups are suggested by hydroxylamine and semicarbazide inhibition. Possible inhibition of succinic dehydrogenase is upheld by the inhibitory effects of fluoride and cyanide. Oxidation of an α -keto acid, which could be α -ketobutyrate, is disclosed by arsenite inhibition. Transamination is indicated by cycloserine, aminooxyacetic acid and thyroxine inhibitions. The lack of

potent inhibition by monofluoroacetate suggested that aconitase activity of the TCA cycle is of minor importance to ethylene production. In fact, the lack of inhibition by malonate coupled with the weak inhibition of monofluoroacetate indicates that a large segment of the TCA cycle is dispensable to ethylene evolution.

Inhibition of the cytochrome system would reduce the synthesis of ATP and since this cofactor is a stimulant to ethylene evolution (97, 155) then inhibition of ethylene production would be expected. The carbonyl groups involved may be those of pyridoxal phosphate in the transaminase, and possibly that of α -ketobutyrate. (According to the scheme outlined in Chapter 3, transamination would be involved in the production of aspartate from glutamate, and propionate from β -alanine.)

Table III. Effect of Various Inhibitors on Ethylene Production
by a Particulate Fraction from Tomatoes

C ₂ H ₄ (μpl)											
Arsenite (Na salt) (10 ⁻⁴ M)				Arsenite (Na salt) (10 ⁻³)				Arsenite (Na salt) (10 ⁻¹ M)			
Amino oxyacetic Acid (10 ⁻³ M)											
Run No.				3				5			
1								7			
Period	A	B	%	A	B	%	A	B	A	B	%
0 - 3 h	171	405	+137	253	169	-33	229	301	287	250	-13
3 - 22 h	N	N		N	N		126	23	73	56	
22 - 24 h	658	624	- 5	851	613	-28	2317	122	2238	1957	-13
Total	829	1029	+24	1104	782	-29	2672	446	2598	2263	-13
Nitrogen (mg)	10.68	10.59		6.42	6.39		13.79	14.91	6.51	6.80	
Run No.				4				6			
2								8			
0 - 3 h	174	191	+10	169	194	+15	96	287	149	96	-36
3 - 22 h	N	N		73	70		68	T	117	73	
22 - 24 h	728	726		496	230	-54	2199	333	812	697	-14
Total	902	927	+ 3	738	494	-33	2363	620	1078	866	-20
Nitrogen (mg)	7.21	7.32		6.48	6.91		15.02	14.70	5.25	5.60	

Table III. (cont'd)

C ₂ H ₄ (m μ l)											
Azide (Na salt) (10 ⁻³ M)				p-CMB (Na salt) (10 ⁻⁴ M)				p-CMB (Na salt) (1 x 10 ⁻³ M)			
A				B				A			
B				B				B			
%				%				%			
Run No.				11				13			
Period				A				A			
0 - 3 h				230				231			
3 - 22 h				77				11			
22 - 24 h				953				590			
Total				1233				832			
Nitrogen (mg)				5.49				5.07			
Run No.				12				14			
0 - 3 h				358				293			
3 - 22 h				77				56			
22 - 24 h				956				464			
Total				1391				813			
Nitrogen (mg)				5.86				5.97			

* 10⁻²M glutathione (GSH) added one hr. after initial sonication.

Table III. (cont'd)

C ₂ H ₄ (m μ l)																			
Cyanide (K salt) (1 x 10 ⁻⁴ M)				Cyanide (K salt) (2 x 10 ⁻⁴ M)				Cyanide (K salt) (10 ⁻² M)				D—Cycloserine (1 x 10 ⁻³ M)							
Run No.				17				19				21				23			
Period		A	B	%	A	B	%	A	B	%	A	B	%	A	B	%			
0 - 3 h		61	118	+93	304	332	+ 9	338	512	+51	242	175	-28						
3 - 22 h		84	T		36	36		619	203		43	17							
22 - 24 h		343	307	-10	1508	1918	+27	866	478	-45	1423	932	-35						
Total		488	425	-13	1848	2286	+24	1373	1203	-12	1708	1124	-34						
Nitrogen (mg)		3.99	4.27		12.34	13.21		10.76	11.55		8.79	10.11							
Run No.				18				20				22				24			
0 - 3 h		362	338	- 7	568	656	+15	276	310	+12	287	253	-12						
3 - 22 h		45	11		141	101		96	101		56	68							
22 - 24 h		1021	940	- 8	2019	2136	+ 6	715	465	-35	1079	623	-42						
Total		1428	1289	-10	2728	2893	+ 6	1087	876	-19	1422	944	-34						
Nitrogen (mg)		6.39	6.13		13.39	13.18		6.83	6.53		7.09	7.09							

Table III. (cont'd)

C ₂ H ₄ (mpul)											
DIECA (Na salt) (5 x 10 ⁻⁴ M)				DIECA (Na salt) (10 ⁻³ M)				Fluoride (Na salt) (10 ⁻³ M)			
								Fluoride (Na salt) (10 ⁻¹ M)			

Table III. (cont'd)

C ₂ H ₄ (m μ l)												
Hydroxylamine HCl (10 ⁻³ M)				Iodoacetamide (10 ⁻⁴ M)				Iodoacetamide (10 ⁻² M)				Malonic Acid (10 ⁻³ M)
Run No.	33			35			37			39		
Period	A	B	%	A	B	%	A	B	%	A	B	%
0 - 3 h	191	186	- 3	154	120	-22	225	169	-25	366	428	+17
3 - 22 h	T	T		9	N		34	28		168	169	
22 - 24 h	945	446	-53	882	517	-41	1052	383	-64	1177	1142	- 3
Total	1136	643	-43	1045	637	-39	1311	580	-56	1711	1739	+ 2
Nitrogen (mg)	8.58	8.84		7.53	8.02		6.74	7.18		6.30	7.65	
Run No.	34			36			38			40		
0 - 3 h	140	271	+94	126	101	-20	164	237	+45	102	175	+72
3 - 22 h	25	N		N	17		101	121		39	135	
22 - 24 h	491	214	-56	790	499	-37	675	388	-43	543	486	-10
Total	656	485	-26	916	617	-33	940	746	-21	684	796	+16
Nitrogen (mg)	9.10	9.01		6.91	7.26		11.32	11.64		6.23	5.53	

Table III. (cont'd)

C ₂ H ₄ (m μ l)											
Malonic Acid (10 ⁻² M)				Mercuric Sulfate (10 ⁻² M)				Monofluoroacetate (Na salt) (10 ⁻⁴ M)			

Table III. (cont'd)

C ₂ H ₄ (m μ l)												
Oxythiamine (10 ⁻³ M)				Semicarbazide HCl (10 ⁻³ M)				Silver Nitrate (10 ⁻³ M)				L-Thyroxine (Na salt) (10 ⁻³ M)
Run No.				51				53				55
Period	A	B	%	A	B	%	A	A	B	%	A	B %
0 - 3 h	140	292	+109	329	253	-23	110	129	129	+17	194	259 +34
3 - 22 h	25	N		17	6		45	62	62		45	25
22 - 24 h	491	658	+34	1287	1023	-21	374	238	238	-36	2430	2256 - 7
Total	656	950	+45	1633	1282	-21	529	429	429	-19	2669	2540 - 5
Nitrogen (mg)	9.10	8.75		6.48	6.74		6.83	6.96	6.96		8.41	8.21
Run No.				52				54				56
0 - 3 h	211	209		277	158	-43	270	73	73	-73	276	306 +11
3 - 22 h	14	65		7	N		11	N	N		51	34
22 - 24 h	675	875	+30	782	709	- 9	400	248	248	-38	2469	2057 -17
Total	900	1149	+28	1066	867	-19	681	321	321	-53	2796	2397 -14
Nitrogen (mg)	7.44	8.05		7.39	7.35		6.83	6.96	6.96		8.19	7.73

Table III. (cont'd)

Basic Reaction Mixture

0.5M sucrose

0.125M KH_2PO_4

pH 7.2

$1.9 \times 10^{-3}\text{M}$ ATP added to each flask after initial sonication. Particles were sonicated for 4 min. at 1.2 amp at the beginning of the collection periods 0-3 hr and 22-24 hr, respectively.

A = control containing particles suspended in basic reaction mixture but without added inhibitor

B = flask containing the same as A, but with added inhibitor

% = percent increase or decrease in ethylene production

T = trace amount

N = none detectable

6. The Effects of Several Cations on Ethylene Production

A study of the effects of several cations was made to determine which cations were stimulatory to ethylene production by the tomato particulate suspension. Quite often, the stimulation by a cation in metabolism of a compound will assist in identifying the enzyme responsible for the metabolism of it. Abeles and Rubenstein (73) have published a preliminary note on the effects of such cations on ethylene production by a pea enzyme preparation.

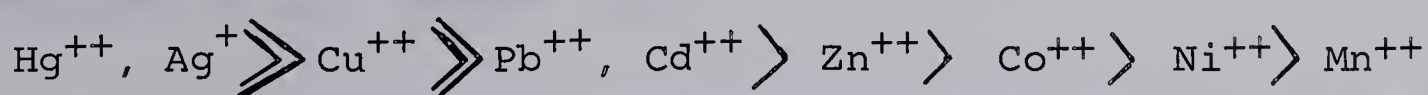
It would appear that a study of this nature is not without its difficulties. The interaction of cations with each other and with enzymes helps to complicate the interpretation of results. There is, also, the endogenous supply of cations that must be considered when evaluating effects of added cations. Both activation and inhibition may result from the addition of one cation. In specific enzyme studies a particular cation will stimulate the enzyme actively until the optimal concentrations has been reached and then it may become severely inhibitory as the concentration exceeds the optimum level. For example, an NAD kinase enzyme from yeast although activated by Mn^{++} and Mg^{++} was strongly inhibited by Mn^{++} and appreciably inhibited by Mg^{++} when optimum levels of either were exceeded (166).

A difficulty encountered with dual cation-activated enzymes is that inhibition may result when one cation is present at the optimal concentration while that of the other cation is suboptimal.

Inhibition of this type occurs with enolase from yeast (166) when Zn^{++} in the presence of Mg^{++} at optimal concentration, behaves in an inhibitory manner.

Formation of an inactive metal-enzyme complex is possible on the addition of cations to an enzyme preparation. This would be pronounced if the added cation had a greater affinity for the enzyme than the activating cation.

Most cations exhibit toxic effects when present in amounts exceeding that normally found in plants and animals. An excess of cobalt in animals results in polycythemia and manganese toxicity in plants produces dark-brown necrotic areas in leaves, petioles and stems. However, the effect of cations in the present study is not that at the physiological level but rather at the enzymic level. Several groups on the protein may bind or chelate with cations. It is estimated that at pH 7.0 a protein has all of its carboxyl groups, half of its imidazole groups, a small fraction of its thiol groups (1%) and a weak fraction of its ϵ -amino groups (0.1%) in the free or dissociated forms. Thiol groups chelate readily with several cations and chelation with this group is probably the primary inhibitory effect of cations. The relative affinities of various cations for thiol groups have been shown to be:

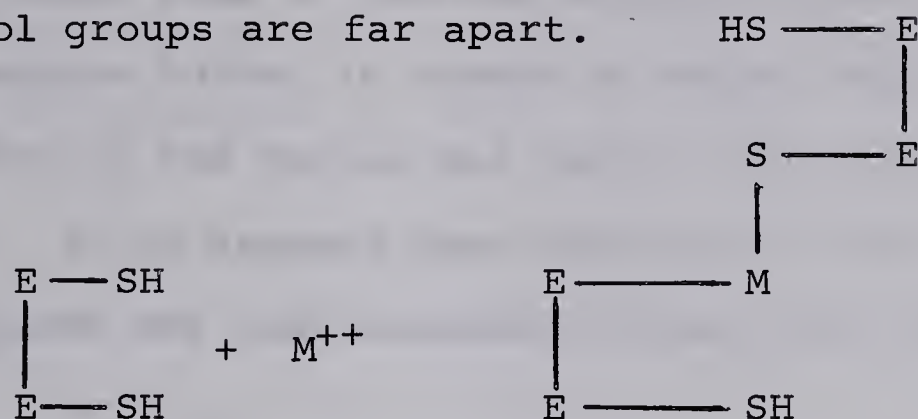


Chelation of the thiol groups may be illustrated as follows:

i) Thiol groups are close together.



ii) Thiol groups are far apart.



A table of values listing the affinities of cations for various groups on the protein will be useful in determining the inhibitory effects of cations.

First Association Constants^m (Log k_i) For the
Combination of Some Cations and Small Molecules^a

Cation	Sulfide	RS ⁻	NH ₃	Imidazole
Hg ⁺⁺	53.5	20	8.8	-
Ag ⁺	50	15	3.2	-
Cu ⁺⁺	41.5	-	4.2	4.4
Co ⁺⁺	26.7	9	-	-
Zn ⁺⁺	25.2	7	2.8	2.6

^aTabulated by N.B. Madsen (166).

It is evident that enzymes may be inactivated by chelation of groups important to the active site and to maintenance of the conformational state.

Results and Discussion

The results of the effects of several cations on ethylene production by a tomato particulate fraction are tabulated in Table I (page 107). Since some of cations studied formed precipitates in the phosphate buffer it cannot be stated what the effective concentration of the cation was during the course of the experiment. It is assumed that chelation of the cation would gradually force the undissociated cation into the dissociated form.

a) Mg^{++} ($1 \times 10^{-3}\text{M}$, added as MgSO_4) : Runs 1, 2

Total ethylene production decreased slightly in the presence of magnesium ions.

The lack of stimulation by Mg^{++} was somewhat surprising in view of its stimulatory effect when TPP was also added (see Chapter 4). However, experiments in the latter study disclosed a stimulatory effect for Mg^{++} when TPP was employed at $2 \times 10^{-3}\text{M}$ but not when the concentration was reduced to $2 \times 10^{-4}\text{M}$. Evidently, the stimulatory effect of Mg^{++} is dependent upon the concentration of TPP. In the absence of an added source the endogenous supply of TPP may have been insufficient to complex with the magnesium ions and thus, stimulate ethylene production.

Lehninger et al (163) found that fragmented mitochondria contain an ample amount of Mg^{++} bound to the membranes. It is possible then that the exogenous source of the cation may have exerted an inhibitory effect resulting from a concentration exceeding the optimal value for the cation.

Abeles and Rubenstein (73) studied a number of cations on ethylene production and found all except Mn^{++} to be either without effect, or inhibitory.

b) Mn^{++} (1×10^{-3} M, as MnSO_4) : Runs 3, 4.

Manganese ions at 10^{-3} M, had little effect on ethylene production by the tomato particulate fraction.

Since no effect was detected with Mn^{++} , little can actually be stated about the cation. Whether varying the concentration would have altered the effect can only be determined by further work. Abeles and Rubenstein (73) observed a slight stimulatory effect on ethylene production by a pea enzyme preparation when Mn^{++} was used at 5×10^{-4} M, but increasing levels of the cation gave progressive inhibition. It is significant to note that a concentration of 10^{-3} M had little effect on ethylene evolution by the tomato particulate fraction. The difference in biochemical properties and behaviors may account for the observed responses of both systems to manganese ions.

c) Fe^{++} (1×10^{-3} M, as FeSO_4) : Runs 5, 6.

Mild inhibition of ethylene evolution by the tomato fraction was observed in the presence of 10^{-3} ferrous ions.

The inhibitory effect of Fe^{++} may be due to its chelation with thiol or other groups on an enzyme essential to ethylene production. An inhibitory effect resulting from its oxidation to Fe^{+++} may be suspected by is unlikely since ferric ions were without effect when added with pyridoxal phosphate to the particulate suspension (see Chapter 4).

d) Cu^{++} (1×10^{-3} M, as CuSO_4) : Runs 7, 8.

Considerable inhibition of ethylene production by the aged, resonicated particles was noted with 10^{-3} Cu^{++} .

The primary inhibitory effect of Cu^{++} is that of chelation with thiol groups. In relative affinity for the group copper ranks just below mercury and silver ions and is therefore, classified as an avid thiol reactant. Succinic dehydrogenase because of its thiol groups is inhibited by Cu^{++} (167). Moreover, from the table presented in the beginning of the chapter it is seen that copper ions will react with amino and imidazole groups and thus, may inactivate the substrate binding sites of the enzyme. The inhibitory effect of copper, if predominantly that of thiol chelation, agrees well with the results obtained with Hg^{++} , Ag^+ , p-CMB and iodacetamide. It is interesting to note that Lehninger et al (163) found Cu^{++} bound to fragmented mitochondria but what consequence this might hold for the result obtained is unknown.

e) Zn^{++} (1×10^{-3} M, as ZnSO_4) : Runs 9, 10.

Total ethylene production was reduced approximately 30% in the presence of 10^{-3} M Zn^{++} .

Since Zn^{++} resembles Cu^{++} in its inhibitory affect on enzymes the reader is referred to the section concerned with copper for a more comprehensive treatment. Because its affinity for specific protein groups is less than that of Cu^{++} , one might expect milder inhibition with Zn^{++} but both cations behaved similarly.

f) Co^{++} ($1 \times 10^{-3}\text{M}$, as CoCl_2) : Runs 11, 12

Marked inhibition of total ethylene production and that for the 22 - 24 hr. period was measured when 10^{-3}M Co^{++} was added to the particulate suspension.

Inhibition by cobalt ions probably results from thiol chelation if the table of relative affinities quoted earlier can be used as the sole criterion. Its relative affinity is close to that of Zn^{++} and thus similar inhibitory trends would be expected. However, the effect with Co^{++} is significantly greater than with Zn^{++} and may indicate that groups other than thiols react with the cation.

g) Al^{+++} ($1 \times 10^{-3}\text{M}$, as $\text{Al}_2(\text{SO}_4)_3$) : Runs 13, 14

Aluminum ions at 10^{-3}M had no obvious effect on ethylene evolution by the tomato particles.

Several amino acid decarboxylases and transaminases require a trivalent cation for maximum enzymic activity. Transamination, which has been implied by the inhibitory effects of cycloserine and aminooxyacetic acid, apparently does not require added trivalent cations since Al^{+++} and Fe^{+++} (see Chapter 4) were without effect.

The inhibitory effects of Cu^{++} , Zn^{++} and Co^{++} are probably valid indications that essential thiol groups are necessary to the biosynthesis of ethylene. What contribution other groups in proteins may have to the biogenesis of ethylene is

a matter for conjecture and further research. The lack of any cation stimulation indicates that either a sufficient concentration is present endogenously or that other factors are required to evolve the stimulatory effect. Exemption of an added trivalent cation for transaminase activity, as suggested in Chapter 4, is verified by the lack of effect with aluminum ions. High sensitivity of the ethylene biosynthetic system to cobalt is suggested by the pronounced inhibitory effect of the cation.

TABLE IV. Effect of Several Cations on Ethylene Production by A Particulate Fraction
From Tomatoes

C ₂ H ₄ (μl.l)													
Mg ⁺⁺ (MgSO ₄) (10 ⁻³ M)			Mn ⁺⁺ (MnSO ₄) (10 ⁻³ M)			Fe ⁺⁺ (FeSO ₄) (10 ⁻³ M)			Cu ⁺⁺ (CuSO ₄) (10 ⁻³ M)				
Run No.		1	3		5		7						
Period	A	B	%	A	B	%	A	B	%				
0 - 3 h	118	88	-25	149	127	-15	155	198	+28	118	99	-16	
3 - 22 h	51	72		17	13		56	45		51	84		
22 - 24 h	1081	857	-21	812	763	- 6	391	359	- 8	1081	507	-53	
Total	1250	1017	-19	1078	903	-16	608	602	-	1250	690	-45	
Nitrogen (mg)	7.44	6.83		5.25	5.60		8.84	8.58		7.44	7.44		
Run No.		2	4		6		8						
0 - 3 h	332	362	+ 9	332	354	+ 7	118	107	- 9	225	298	+32	
3 - 22 h	45	N		45	N		51	68		90	54		
22 - 24 h	1395	1193	-14	1395	1361	- 2	1081	676	-37	551	382	-31	
Total	1772	1555	-12	1772	1715	- 3	1250	851	-32	866	734	-15	
Nitrogen (mg)	6.74	6.67		6.74	5.86		6.04	5.92		4.99	4.90		

TABLE IV (Cont'd)

C ₂ H ₄ (mμl)									
Zn ⁺⁺ (ZnSO ₄) (10 ⁻³ M)				Co ⁺⁺ (CoCl ₂) (10 ⁻³ M)			Al ⁺⁺⁺ (Al ₂ SO ₄) ₃ (10 ⁻³ M)		
Run No.	9			11			13		
Period	A	B	%	A	B	%	A	B	%
0 - 3 h	87	104	+20	176	115	-35	176	141	-20
3 - 22 h	59	7		15	17		15	N	
22 - 24 h	1405	900	-36	638	362	-43	638	627	- 2
Total	1551	1011	-35	829	494	-40	829	768	- 7
Nitrogen (mg)	7.79	8.09		10.90	10.47		10.90	10.85	
Run No.	10			12			14		
0 - 3 h	250	194	-22	155	160	+ 3	135	207	+34
3 - 22 h	N	9		56	34		56	47	
22 - 24 h	1690	1238	-27	391	110	-72	391	409	+ 5
Total	1940	1441	-26	602	304	-49	602	663	+10
Nitrogen (mg)	8.40	8.41		8.84	8.75		8.84	8.93	

Basic Reaction Mixture
-0.5 M sucrose
-0.125 M KH_2PO_4
pH 7.2
 1.9×10^{-3} ATP added
to each flask after
initial sonication.

Particles were sonicated for 4 min. at 1.2 amp at the beginning of collection periods 0-3 hr and 22-24 hr, respectively.

T = trace amount

N = none detectable

A = control containing particles suspended in basic reaction mixture but without added cation.

B = flask containing the same as A, but with added cation.

% = percent increase
or decrease in ethyl-
ene production.

7. Effects of pH on Ethylene Production

The behavior of the ethylene producing system in the tomato particulate fraction was studied within a fairly wide range of pH values. Determination of a pH optimum was not attempted but increments of one pH unit within the range of 3.0 to 9.0 were employed and their effect on ethylene production was compared to controls at a pH of 7.0.

That enzymic activity is profoundly influenced by pH is a firmly established fact and cursory treatment of the subject at this moment would be superfluous. A comprehensive review of the topic is available in the text by Dixon and Webb (192).

Quite often, pH effects on a biological system enable one to suspect the activity of individual enzymes or of a group of enzymes. This is especially feasible in a simplified system where the number of enzymes is limited. However, a particulate fraction contains an extensive enzyme complex, and it becomes difficult to make tentative identification of enzymes on the basis of pH effects.

The effects of pH on ethylene production by the tomato fraction may permit a better understanding of the climacteric and post-climacteric periods of a fruit. In this respect, it may provide some insight into the integration of the biochemical activities of the soluble phase and particulate phase insofar as ethylene biosynthesis is concerned and also assist in elucidating the interrelationships of ethylene production, pH and other biochemical events during the two periods.

Results and Discussion:

The results of the experiments on the effects of pH on ethylene production are presented in Table V (page 113). It should be pointed out that although particles of the controls were suspended in a buffer of pH 7.2, the pH at the end of the run (after 24 hours of ethylene collection) was 7.0. Furthermore, the pH values of 8.0 and 9.0 were achieved with Tris buffer (0.125 M Tris-hydroxyaminomethane, 0.5 M sucrose) as the phosphate buffer failed to maintain these values.

a) pH 3.0 : Runs 1, 2

Initial production of ethylene was higher at pH 3.0 than at pH 7.0. However, production during the 22-24 hr. period was reduced by more than 50% when a pH of 3.0 was used.

The observation that initial production was not altered by the low pH value suggests that ethylene evolved during this period may be preformed in the particles and is liberated upon disintegration of the particles. Extensive denaturation of many enzymes at the pH of 3.0 probably accounts for the pronounced reduction of ethylene production upon ageing of the suspension.

b) pH 5.0 : Runs 3, 4

Total ethylene evolution was weakly stimulated when a pH of 5.0 was employed.

The weak stimulation observed would almost imply that a probable pH optimum for the ethylene producing system is at a value less than 7.0. However, one cannot strictly speak of pH optimum for the tomato fraction because the ethylene biosynthetic sequence is not devoid of the influence of other enzymes. A more appropriate reference is in

terms of values that are more effective in promoting ethylene production.

c) pH 6.0: Runs 5, 6

Production of ethylene during the 22-24 hr. collection period was augmented when the tomato particulate fraction was subjected to a pH of 6.0.

The increase in ethylene evolution at pH 6.0 supports the contention that an acidic medium is apparently more effective than a neutral one in elevating the activity of the system synthesizing the volatile.

d) pH 8.0: Runs 7, 8

Considerable inhibition of ethylene production was observed at pH 8.0.

The decrease in ethylene evolution discloses an obvious sensitivity of the enzymic system to an alkaline pH. It is interesting to speculate whether the inhibition is a result of inactivation of the enzymic state (altered conformation), unavailability of a substrate because of its change of ionic form, or inactivation of a cofactor associated with an enzyme.

e) pH 9.0: Runs 9, 10

Total production of ethylene decreased 45% at pH 9.0 and a lighter red color was also noticed with the particulate suspension.

The comments made for the results obtained with pH 8.0 are entirely applicable to the results observed at pH 9.0 except that inhibition is more extensive at the

latter pH value.

Abeles and Rubenstein (73) observed a pH optimum of 4.5-4.7 for a pea enzyme preparation. The comment made earlier that the more complex system in the tomato fraction may not have a well defined pH optimum is certainly substantiated by the results obtained with pH values of 5.0 and 6.0, as both stimulated the production of ethylene. Thompson and Spencer (74) found that a purified enzyme preparation from bean cotyledons catalyzed production of ethylene with the pH range of 6.7-7.2. The differences in the values observed for the three studies amply indicate the variance of different preparations in sensitivity to pH effects, with the purified system being the most sensitive of the three.

It is interesting to note that a "substrate" isolated by Abeles and Rubenstein (73) from the pea homogenate was completely inactivated at pH 9.0 with regards to the enzymic production of ethylene from it. Since TPP was shown to stimulate ethylene synthesis in the tomato fraction, the postulation offered is that the "substrate" isolated may have contained TPP and subjecting to an alkaline medium may have destroyed the co-factor. The inactivation of TPP may be a primary cause for the pronounced reduction in ethylene production by the tomato particulate fraction at alkaline pH values. However, other probable causes include formation of ionic forms of enzymes that are inactive or ionic forms of substrates that are not readily metabolized.

Table V. The Effects of pH on Ethylene Production by a Particulate Fraction From Tomatoes

C ₂ H ₄ (mμl)										
pH 3.0			pH 5.0			pH 6.0				
Run No.	1		3		5					
Period	A	B	%	A	B	%	A	B	%	
0 - 3 h	264	293	+11	374	396	+ 6	374	216	-42	
3 - 22 h	56	73		20	42		20	25		
22 - 24 h	664	246	-63	495	602	+22	495	649	+31	
Total	984	612	-38	889	1040	+17	889	890	-	
Nitrogen (mg)	8.93	8.58		8.72	8.26		8.72	8.14		
Run No.	2		4		6					
0 - 3 h	187	270	+44	253	276	+ 9	253	259	+ 2	
3 - 22 h	42	73		51	54		51	51		
22 - 24 h	508	211	-58	497	556	+12	497	551	+11	
Total	737	554	-25	801	887	+11	801	861	+ 7	
Nitrogen (mg)	7.00	6.48		9.07	8.40		9.07	8.05		

TABLE V (Cont'd)

C ₂ H ₂ (mμl)		pH 8.0		pH 9.0	
Run No.		7	9		
Period	A	B	%	A	B
0 - 3 h	203	197	-3	264	287
3 - 22 h	N	N		56	45
22 - 24 h	394	248	-37	664	298
Total	597	445	-25	984	530
Nitrogen (mg)	8.58	8.58		8.93	8.85
Run No.	8	10			
0 - 3 h	323	255	-21	323	317
3 - 22 h	39	47		39	38
22 - 24 h	899	482	-46	899	330
Total	1261	784	-38	1261	695
Nitrogen (mg)	7.35	7.76		7.35	7.55

I. Basic Reaction Mixture for Neutral and Acidic pH Values:

0.5 M sucrose
0.125 M KH₂PO₄
1.9 x 10⁻³M ATP added to each flask after the initial sonication.

II. Basic Reaction Mixture for Alkaline pH Values:

0.5 M sucrose
0.125 M Tris (tris-hydroxyaminoethyl-methane)
1.9 x 10⁻³ M ATP added to each flask after the initial sonication.

Particles were sonicated for 4 min at 1.2 amp at the beginning of the 0-3 hr and 22-24 hr collection periods.

T = Trace amount

N = None detectable

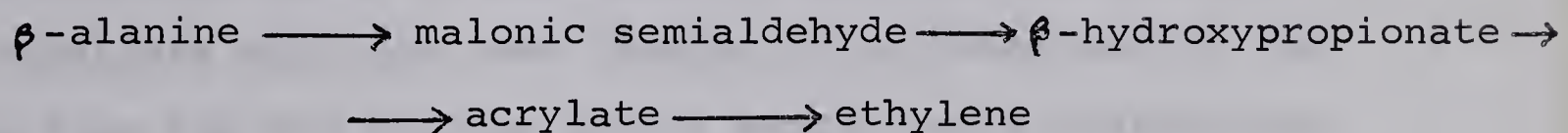
A = Control containing particles suspended in basic reaction mixture (I) adjusted to pH 7.2.

B = Flask containing particles suspended in basic reaction mixture I or II adjusted to designated pH value.

% = percent increase or decrease in ethylene production.

8. Effects of Cofactors and Substrates with β -Alanine on Ethylene Production

It was previously mentioned that a tentative scheme for ethylene synthesis from β -alanine was proposed by Thompson and Spencer(74). The postulated pathway was based on results obtained from tracer studies with β -alanine. When β -alanine -2-C¹⁴ was administered to a bean enzyme preparation, labelled ethylene was detected and intermediates identified in the pathway were also found to contain label. The pathway again is,



Wang et al (54) also observed labelling of ethylene from β -alanine by Penicillium digitatum and thus confirmed the result of Thompson and Spencer.

Since β -alanine arises from nucleic acid catabolism in plants (144, 145) an important relationship may, therefore, exist between the two. Pertinent to this argument is the observation that net protein content increases during the climacteric period of fruits (105,106,107). Consequently, one would expect an increase in the RNA level and also an increase in the nucleic acid pool during this period. That increased ethylene production should be concomitant event is not improbable.

Preliminary experiments with β -alanine revealed that added cofactors were required to stimulate conversion of the amino acid to ethylene. Subsequently, a study on the effects of several cofactors on β -alanine metabolism to ethylene was completed. In addition, two other studies involving partially reconstituted decarboxylation systems and β -alanine were also completed but these will be discussed individually.

A difficulty in the interpretation of the results is deciding whether the effect of a cofactor was direct or indirect. Since a previous study on cofactors in the absence of any added substrate was done (see Chapter 4) a comparison of the results from the two studies should enable valid distinction between a direct or an indirect effect.

Results and Discussion

The results of the effects of several cofactors on β -alanine metabolism to ethylene are presented in Table VI (Page 123). Addition of β -alanine and cofactors was done after initial sonication of the suspension. The concentration of β -alanine was 0.05M.

a) Coenzyme A (7×10^{-5} M) : Runs 1, 2

Total ethylene production decreased slightly when catalytic amounts of CoA were added to the suspension.

The result is comparable to the one obtained when the cofactor alone was studied. It would appear that acylation of an intermediate by CoA is detrimental to ethylene production.

A possible explanation is that some acrylate in the presence of CoA forms acrylyl CoA and proceeds to β -alanyl CoA by amination or transamination rather than undergoing decarboxylation to ethylene. Amination of acrylyl CoA has been reported by Vagelos, Earl and Stadtman (193) with a bacterial enzyme. Since the enzyme is stimulated by a medium of high ionic strength (up to 9.0) it is unlikely that amination of acrylyl CoA proceeds at a significant rate.

b) Flavin Adenine Dinucleotide ($1.2 \times 10^{-4}M$) : Runs 3,4

A slight increase in ethylene production was observed when FAD (0.1 mg/ml) was added to the particulate suspension.

It is interesting to note that the cofactor in the absence of β -alanine caused a significant decrease in the production of ethylene for the 22 - 24 hr. period (see Table II) but in the presence of the amino acid a slight increase was observed for the same period. It is possible that in the presence of β -alanine the cofactor (in its reduced form) could function in the reduction of malonic semialdehyde (derived from the deamination of β -alanine) to form β -hydroxypropionate, and that in the absence of the amino acid reduced FAD was not oxidized readily and thus may have reduced the rate of formation of acrylate. In Chapter 4, it was suggested that a complex between FAD and an intermediate, which if not acted upon, could be responsible for the decrease in ethylene evolution. It is conceivable then, that β -alanine indirectly assisted ,

in the manner described above, in dissociating the complex into oxidized FAD and a dehydrogenated intermediate.

c) Flavin Mononucleotide ($2 \times 10^{-4}M$) : Runs 5, 6

The comments made for FAD are also applicable to an interpretation of the results with FMN, since both flavin coenzymes behave similarly in biological systems.

d) α -Ketoglutaric Acid ($1 \times 10^{-3}M$) : Runs 7,8

The effect of $10^{-3}M$ α -ketoglutaric acid on ethylene production was slight.

Since the first step in the conversion of β -alanine to ethylene is a transamination reaction then the addition of an amino group acceptor like α -ketoglutaric acid should stimulate the conversion of β -alanine to the olefin. However, α -ketoglutaric acid had but a minor effect that may be a resultant of weak metabolism of β -alanine, or a high degree of specificity for the amino group acceptor, or the presence in the preparation of saturating amounts of α -ketoglutaric acid.

e) α -Lipoic acid ($1 \times 10^{-3}M$) : Runs 9, 10

Total ethylene production was weakly increased on addition of $10^{-3}M$ α -lipoic acid.

The increase obtained with α -lipoic acid and β -alanine is of the same magnitude as that realized from α -lipoic acid alone. It is doubtful that an effect has been exerted on the amino acid by the cofactor. Consequently, a role for oxidative

decarboxylation in ethylene biogenesis is not evident or perhaps other cofactors must be present before α -lipoic acid can give its maximum stimulatory effect.

f) Nicotinamide Adenine Dinucleotide ($3 \times 10^{-4}M$) : Runs 11, 12

Addition of NAD and β -alanine to the suspension resulted in a decrease in the yield of the volatile.

An explanation for the behavior of NAD on ethylene production by the particulate fraction has been given in Chapter 4. The results with NAD in the present study appear to support the conviction that an NAD linked dehydrogenase is not involved in ethylene biosynthesis. Whenever a cofactor fails to be stimulatory to ethylene production one can cite at least three reasons for this behavior, first, that the cofactor has no role; second, that it is already present in ample amounts; and third, that some other factors are lacking in the enzyme system. Which reason explains the behavior of NAD cannot be identified on the results from this experiment.

g) Nicotinamide Adenine Dinucleotide Phosphate ($4 \times 10^{-4}M$)
Runs 13, 14

The total output of ethylene was augmented in the presence of $4 \times 10^{-4}M$ NADP.

The increase corresponds to the effect observed when the cofactor by itself was added to the system (Table II). It is doubtful that NADP had any pronounced effect on β -alanine metabolism to ethylene. This may mean that sufficient NADP is

present in the suspension or that the cofactor is assisting ethylene production in some indirect manner. One could probably resolve the behavior of the cofactor by observation of the effects of administration of an analogue of the nucleotide.

h) Oxalacetic acid ($1 \times 10^{-3}M$) : Runs 15, 16

Ethylene production was elevated about 10% in the presence of $10^{-3}M$ oxalacetic acid.

The increase observed with oxalacetic acid is similar to that obtained with α -ketoglutaric acid. Two possibilities may account for the weak stimulatory effect of oxalacetic acid, first, that transamination proceeds slowly, and secondly, that the transaminase may be highly specific in its requirement for a carboxylic acid as the amino group acceptor. A saturating amount of oxalacetic acid may also be present in the preparations.

i) Oxythiamine ($1 \times 10^{-3}M$) : Runs 17, 18

A stimulatory effect on ethylene evolution was noticed with $10^{-3}M$ oxythiamine.

Although the inhibitor seriously inactivates α -carboxylase activity it did not alter the metabolism of β -alanine. It is possible that oxythiamine inhibits another enzyme nonconducive to ethylene synthesis or it may function in a manner totally unsuspected or unknown.

j) Pyridoxal Phosphate ($5 \times 10^{-4}M$) : Runs 19, 20, 21, 22

A significant increase in total ethylene production was observed when $5 \times 10^{-4}M$ pyridoxal phosphate was added to the particulate suspension. Addition of $10^{-4}M$ Fe^{+++} was without effect in the presence of pyridoxal phosphate.

Pyridoxal phosphate in the absence of an added substrate failed to exhibit any effect on ethylene production but when β -alanine was also added a small stimulation in the production by the cofactor was recorded. It is likely that transamination occurred with β -alanine to form malonic semialdehyde. An endogenous supply of pyridoxal phosphate may be responsible for the weak stimulatory effect of the cofactor on β -alanine and also for the lack of an effect in the absence of an added substrate. The failure of Fe^{+++} to give a further increase in ethylene production reaffirms the suggestion that a trivalent cation is not essential to the transamination reaction.

k) Thiamine Pyrophosphate ($2 \times 10^{-3}M$) : Runs 23,24,25,26

The addition of TPP helped to increase production of the olefin by 10%. When $10^{-3}M$ Mg^{++} was also present, a further increase of 20% was obtained.

The results found with both cofactors and β -alanine are comparable to those when β -alanine was not added to the suspension. Therefore, it appears that β -alanine metabolism to ethylene was not enhanced by the addition of TPP and Mg^{++} .

Perhaps a rate limiting step in β -alanine metabolism to ethylene exists and thus, stimulatory effects by adding other cofactors may be limited. There is also the possibility that the concentration of β -alanine may have been high enough to induce competitive inhibition of an enzyme in the metabolic sequence leading from β -alanine to ethylene. Competition between β -alanine and an enzyme for acrylate may, for instance, repress the formation of ethylene. This is not unreasonable because acrylic acid is an avid acceptor of amino groups.

The decrease in ethylene evolution caused by CoA was suggested as a result of an acylated intermediate undergoing a reaction not leading to ethylene. Slight stimulatory effects by the flavin coenzymes were taken to mean that β -alanine assisted in their oxidation, a process that may not have been accomplished readily. With regards to the nucleotide effects, it still appears that NADP may have some function in ethylene biogenesis and that NAD has no similar role. The slight stimulating effect of α -lipoic acid cannot be used to identify the presence of oxidative decarboxylation. Furthermore, it may act as α -lipoyl dehydrogenase exclusive of oxidative decarboxylation (158). How oxythiamine assists in ethylene evolution is open to speculation. The slight increase realized with pyridoxal phosphate reaffirms the need for transamination. Finally, the fact that TPP and Mg did not enhance β -alanine metabolism may be evidence of a rate limiting step not dependent of these cofactors.

Table VI. Effect of Several Cofactors and Substrates with β -Alanine on Ethylene Production by a Particulate Fraction from Tomatoes

C ₂ H ₄ (mμl)																			
Coenzyme A (7 x 10 ⁻⁵ M)				Flavin Adenine Dinucleotide (1.2 x 10 ⁻⁴ M)				Flavin Mononucleotide (2 x 10 ⁻⁴ M)				α-Ketoglutaric Acid (1 x 10 ⁻³ M)							
Run No.				1				3				5				7			
Period		A	B	%	A	B	%	A	B	%	A	B	%	A	B	%			
0 - 3 h		149	180	+21	153	113	-26	152	169	+11	309	324	+ 5						
3 - 22 h		N	N		17	28		N	84		39	12							
22 - 24 h		923	795	-14	744	809	+ 9	1271	1324	+ 4	1942	2098	+ 8						
Total		1072	975	- 9	914	950	+ 4	1423	1577	+11	2290	2332	+ 2						
Nitrogen (mg)		5.25	5.08		4.98	5.34		4.39	3.85		6.65	6.44							
Run No.				2				4				6				8			
0 - 3 h		152	132	-13	402	411	+ 2	153	192	+25	231	245	+ 6						
3 - 22 h		N	N		38	53		17	45		113	96							
22 - 24 h		1271	1220	- 4	660	678	+ 3	744	721	- 3	842	948	+13						
Total		1423	1352	- 5	1100	1142	+ 4	914	958	+ 5	1186	1288	+ 9						
Nitrogen (mg)		4.39	4.25		7.53	7.30		4.98	5.60		6.63	6.33							

Table VI. (cont'd)

C ₂ H ₄ (mμl)																
α-Lipoic Acid (1 x 10 ⁻³ M)				Nicotinamide Adenine Dinucleotide (3.0 x 10 ⁻⁴ M)				Nicotinamide Adenine Dinucleotide Phosphate (4.0 x 10 ⁻⁴ M)				Oxalacetic Acid (1 x 10 ⁻³ M)				
Run No.		9		11		13		15								
Period		A	B	%	A	B	%	A	B	%	A	B	%			
0 - 3 h		167	163	- 2	167	182	+ 9	153	141	- 8	309	259	-16			
3 - 22 h		59	43		59	39		17	14		39	50				
22 - 24 h		1393	1474	+ 6	1393	1082	-22	744	885	+19	1942	2169	+12			
Total		1619	1680	+ 4	1619	1303	-20	914	1140	+25	2290	2478	+ 8			
Nitrogen (mg)		4.54	5.33		4.54	4.73		4.98	6.13		6.65	5.34				
Run No.		10		12		14		16								
0 - 3 h		233	188	-19	197	200	- 2	402	345	-14	169	226	+34			
3 - 22 h		141	48		25	N		38	87		106	15				
22 - 24 h		504	753	+49	783	733	- 6	660	739	+12	1469	1642	+12			
Total		878	989	+13	1005	933	- 7	1100	1171	+ 6	1744	1883	+ 8			
Nitrogen (mg)		4.45	4.21		4.90	2.45		7.53	8.05		4.64	3.68				

Table VI. (cont'd)

C ₂ H ₄ (mμl)																			
Oxythiamine (1 x 10 ⁻³ M)				Pyridoxal Phosphate (5 x 10 ⁻⁴ M)				Pyridoxal Phosphate* (5 x 10 ⁻⁴ M) Fe ⁺⁺ (1 x 10 ⁻³ M)				Thiamine Pyrophosphate (2 x 10 ⁻³ M)							
Run No.				17				19				21				23			
Period				A	B	%	A	B	%	A	B	%	A	B	%				
0 - 3 h				154	210	+36	309	332	+ 7	141	135	- 4	118	138	+17				
3 - 22 h				36	54		39	77		N	N		84	12					
22 - 24 h				1059	1053	- 1	1942	2491	+28	1204	1242	+ 3	704	845	+20				
Total				1249	1317	+ 5	2290	2900	+27	1345	1377	+ 2	906	995	+10				
Nitrogen (mg)				7.09	6.74		6.65	7.00		6.21	5.69		3.12	3.12					
Run No.				18				20				22				24			
0 - 3 h				197	189	- 4	231	219	- 5	100	96	- 4	191	143	-25				
3 - 22 h				25	23		113	101		N	N		N	6					
22 - 24 h				783	1155	+48	842	980	+16	345	336	- 3	309	394	+28				
Total				1005	1367	+36	1186	1300	+10	445	432	- 3	500	543	+ 9				
Nitrogen (mg)				4.90	5.60		6.63	6.45		5.25	6.35		5.34	4.48					

* control also contains 5 x 10⁻⁴M pyridoxal phosphate

Table VI. (cont'd)

C ₂ H ₄ (m μ l)			
Thiamine Phosphate** (2 x 10 ⁻³ M) Mg ⁺⁺ (1 x 10 ⁻³ M)			
Run No.	25		
Period	A	B	%
0 - 3 h	304	360	+18
3 - 22 h	84	28	
22 - 24 h	1237	1711	+38
Total	1625	2099	+29
Nitrogen (mg)	9.54	9.54	
Run No.	26		
0 - 3 h	448	507	+13
3 - 22 h	59	23	
22 - 24 h	1098	1537	+40
Total	1605	2067	+29
Nitrogen (mg)	7.26	6.84	

** control also contains 2 x 10⁻³M TPP

Basic Reaction Mixture

0.5M sucrose

0.125M KH₂PO₄

pH 7.2

0.05M β -alanine

1.9 x 10⁻³M ATP were added to

each flask after initial sonication

Particles were sonicated for 4

min. at 1.2 amp at the beginning

of the 0-3 hr. and 22-24 hr. col-

lection periods, respectively.

A = control containing particles
suspended in basic reaction
mixture, but without added
constituent

-126

B = flask containing the same as
A but with added constituent

% = percent increase or decrease
in ethylene production

T = trace amount

N = none detectable

9. Effects of Cofactors and Substrates with β -Alanine
Decarboxylation System I on Ethylene Production

The purpose of this study and the following one was to attempt a reconstruction of either an enzyme sequence or an enzyme complex or both that would allow maximum stimulation of the conversion of β -alanine to ethylene. This approach is somewhat analogous to the efforts of Sanadi and his co-workers (194, 195) in identification of the components of α -ketoglutaric dehydrogenase. Cofactors found with the latter enzyme were α -lipoic acid, thiamine pyrophosphate, magnesium ion, a flavin nucleotide and a pyridine nucleotide. The complex is arranged so as to allow rapid passage of electrons from one entity to another by virtue of a gradient potential drop.

The author is well aware that concentrations of the various cofactors may not be optimal but it is hoped that deleterious effects do not occur. In this respect, synergistic effects among cofactors may be disrupted or impaired should a deleterious effect become evident.

The β -alanine decarboxylation system in the present study consists of the usual tomato particulate fraction suspended in buffer, with ATP, 0.05M β -alanine, 2×10^{-3} M (or 1mg/ml) thiamine pyrophosphate and 10^{-3} M magnesium ions, to which was added a constituent. The control vessel contained the same materials mentioned, but lacked the additional constituent.

Results and Discussion

The results of the experiments are given in Table VI (page 135).

a) Coenzyme A ($7 \times 10^{-5} \text{M}$) :Runs 1, 2

Ethylene production decreased in the presence of $7 \times 10^{-5} \text{M}$ CoA.

Since this result agrees with those from other experiments employing CoA as a cofactor, the reader is, therefore, referred to Chapters 4 and 8 for an explanation of the inhibitory behavior of the coenzyme in the tomato particulate fraction.

b) Flavin Adenine Dinucleotide ($1.2 \times 10^{-4} \text{M}$) :Runs 3, 4

A significant reduction in evolution of the volatile occurred when FAD was added to the particulate fraction.

The result is contradictory to that obtained with FAD in the presence of β -alanine (Table VI); the latter resulted in a small increase in ethylene production. Two events may be responsible for the reversed effect. First, there may be competition between TPP and FAD for an intermediate or secondly, FAD may produce an oxidized intermediate that cannot be decarboxylated. Another possibility is the diversion of an intermediate to some other product. The likelihood of cofactor antagonism cannot be overlooked but how it would be expressed is unknown.

c) Flavin Mononucleotide ($2 \times 10^{-4}M$) : Runs 5, 6

The results with FMN although somewhat inconsistent in magnitude indicate an increase in the total production of ethylene in the presence of this factor.

The effect of FMN on ethylene production is contradictory to that obtained with FAD. A significant decrease in ethylene evolution was observed with FAD whereas FMN augmented production. Apparently, the deleterious effect on ethylene production of FAD in the presence of TPP and Mg^{++} does not occur with FMN. It would almost appear that an enzyme complex formed with FAD was not as active in ethylene production as a complex containing FMN. The fact that FMN augmented ethylene production in this experiment as well as in a previous study (see Chapter 8) suggests that an FMN containing enzyme is involved in the biosynthesis of ethylene.

d) α -Ketoglutaric acid ($1 \times 10^{-3}M$) : Runs 7,8

The addition of α -ketoglutarate was essentially without effect on the production of ethylene.

The need of an amino group acceptor in the transamination reaction for β -alanine apparently is not met by the added α -ketoglutaric acid as this is the second instance for which the α -keto acid failed to influence ethylene production (see Table VI)

e) α -Lipoic Acid ($1 \times 10^{-3} \text{M}$) : Runs 9, 10

An appreciable increase was observed in total ethylene production on addition of 10^{-3}M α -lipoic acid.

The stimulatory effect of α -lipoic acid realized in this experiment exceeds that of previous studies (see Chapters 4 and 8). Since both thiamine pyrophosphate and α -lipoic acid are present in the suspension, it is highly possible that lipoamide, a factor that participates in decarboxylations, is formed. However, whether formation of this factor favors oxidative or non-oxidative decarboxylation cannot be stated. Oxidative decarboxylation does not appear to be the immediate process because CoA, which is a necessary cofactor in the process, inhibited ethylene production (see Chapter 4 and 8).

f) Nicotinamide Adenine Dinucleotide ($3 \times 10^{-4} \text{M}$): Runs 11,12

Little effect on ethylene evolution was exhibited by NAD at $3 \times 10^{-4} \text{M}$.

It appears that NAD has no major role in the biosynthesis of ethylene. Studied as a cofactor alone (see Chapter 4) and in conjunction with β -alanine (see Chapter 8) NAD failed to stimulate production of the olefin. Inconsequential as exogenous NAD may seem it is entirely too reasonable to assume that an endogenous supply may be more than adequate for normal function in the suspension during the synthesis of ethylene.

- g) Nicotinamide Adenine Dinucleotide Phosphate (4×10^{-4} M)
Runs 13, 14

Stimulation of total ethylene production amounting to 15% was observed when 4×10^{-4} M NADP was added to the suspension.

The increase in ethylene evolution obtained with NADP is approximately of the same value as in the previous studies (see Chapter 4 and 8). However, it is entirely possible that an enzyme complex containing NADP as a moiety was not fully reconstructed in any of these systems and thus, the maximum stimulatory effect latent with NADP could not be expressed.

- h) Oxalacetic acid (1×10^{-3} M) : Runs 15, 16

A significant increase in the yield of ethylene was recorded when oxalacetic acid at 10^{-3} M was added as a cofactor to the decarboxylation system.

The pronounced increase observed with oxalacetic acid must be regarded carefully as several interpretations of this result are evident. In a previous study (Table VI) addition of oxalacetic acid caused only a slight increase in the metabolism of β -alanine to ethylene. However, on addition of TPP and Mg^{++} the dicarboxylic acid elevated ethylene production significantly. In the presence of TPP, Mg^{++} and oxalacetic acid increased decarboxylation of acrylate might occur and transamination, because of an available amino group acceptor, might also be accelerated. Since α -ketoglutaric acid was less effective (Runs 7 and 8), one would conjecture that the

transaminase was more specific for oxalacetic acid.

In the presence of TPP and Mg^{++} oxalacetic decarboxylase activity may be stimulated and pyruvate would be formed. Perhaps pyruvate acid acts as the preferential amino acceptor over oxalacetic and α -ketoglutaric acid. The transamination product would be α -alanine, an amino acid found to promote ethylene production in Penicillium digitatum (40). However, a preliminary experiment showed α -alanine to be considerably less effective than β -alanine as a substrate for ethylene in the tomatoe particulate suspension. Cofactors included in the reaction were TPP and Mg^{++} .

Oxalacetic acid can also accept protons from NAD and NADP linked dehydrogenases and in this manner may promote the formation of an immediate precursor for ethylene from β -alanine. Acceleration of acrylate formation might be a likely consequence of proton acceptance by oxalacetic acid.

i) Oxythiamine ($1 \times 10^{-3}M$) : Runs 17, 18

Ethylene production was weakly increased in the presence of oxythiamine at $10^{-3}M$.

The behavior of oxythiamine is not understood in the biosynthesis of ethylene. Normally, it should inhibit the carboxylase activity proposed in Chapter 3. What can be presumed is that oxythiamine is not acting as oxythiamine pyrophosphate, because the latter acts as a competitive

inhibitor of α -carboxylase and transketolase. More knowledge about the chemistry and biochemistry of the compound may be conducive to finding a satisfactory explanation for the stimulatory effect.

j) Pyridoxal Phosphate ($5 \times 10^{-4}M$) : Runs 19, 20

Total ethylene production decreased slightly in the presence of $5 \times 10^{-4}M$ pyridoxal phosphate.

Why pyridoxal phosphate should exert a slight stimulatory effect with β -alanine and not with the amino acid when TPP and Mg^{++} were also added is difficult to understand. This inconsistent effect of the prosthetic group may indicate the variance in endogenous sources of it. Pyridoxal phosphate may be reacting with amino acids in a non-enzymic manner and may, therefore, reduce the supply of an intermediate if the complex formed did not dissociate. Such an interaction between pyridoxal phosphate and amino acids has been reported by Lucas, King and Brown (196).

For a summary, the results of the effects of cofactors on system I will be compared with those on β -alanine and the tomato particulate system. The decrease in production of ethylene induced by CoA remained unchanged. The slight increase in ethylene evolution observed with added FAD and β -alanine was altered to a significant decrease in system I. The slight stimulatory effect of FMN was evident in both instances. Both amino group acceptors, α -ketoglutaric acid

and oxalacetic acid gave increases in ethylene evolution although the effect of oxalacetic acid was more pronounced in system I. NAD, again, was essentially without effect and NADP retained its slight stimulatory influence. The pyruvic oxidation factor, α -lipoic acid, repeated its weak augmentation of ethylene production as did oxythiamine. Pyridoxal phosphate, which gave an increase with β -alanine, was found to exert little effect in system I.

Table VII. Effects of Cofactors and Substrates with β -Alanine Decarboxylation System I on Ethylene Production by a Particulate Fraction from Tomatoes

C ₂ H ₄ (m μ l)											
Coenzyme A (7 x 10 ⁻⁵ M)			Flavin Adenine Dinucleotide (1.2 x 10 ⁻⁴ M)			Flavin Mononucleotide (2 x 10 ⁻⁴ M)			α -Ketoglutaric Acid (1 x 10 ⁻³ M)		
Run No.			1			3			5		
Period			A	B	%	A	B	%	A	B	%
0 - 3 h			146	158	+ 8	169	200	+18	137	174	+27
3 - 22 h			23	17		88	96		9	135	
22 - 24 h			1603	1300	-19	830	677	-18	586	872	+49
Total			1772	1475	-17	1087	973	-10	732	1181	+61
Nitrogen (mg)			4.64	3.76		5.40	5.72		7.65	8.00	
Run No.			2			4			6		
0 - 3 h			234	234		146	113	-23	268	377	+41
3 - 22 h			96	84		11	11		141	124	
22 - 24 h			860	792	- 8	900	558	-38	626	621	
Total			1190	1110	- 7	1057	682	-35	1035	1122	+ 8
Nitrogen (mg)			6.39	5.51		3.57	4.23		5.03	4.50	

Table VII. (cont'd)

C ₂ H ₄ (mp1)																									
α-Lipoic Acid (1 x 10 ⁻³ M)				Nicotinamide Adenine Dinucleotide (3.0 x 10 ⁻⁴ M)				Nicotinamide Adenine Dinucleotide Phosphate (4.0 x 10 ⁻⁴ M)				Oxalacetic Acid (1 x 10 ⁻³ M)													
Run Nō.				9				11				13				15									
Period		A		B		%		A		B		%		A		B		%							
0 - 3 h		186		124		-37		236		256		+ 8		234		242		+ 3		309		568		+34	
3 - 22 h		N		12				28		28				96		101				75		115			
22 - 24 h		868		1114		+28		2003		2111		+ 5		860		952		+11		1457		2249		+54	
Total		1054		1250		+19		2267		2395		+ 6		1190		1295		+ 9		1841		3132		+70	
Nitrogen (mg)		7.00		8.31				6.56		6.07				6.39		5.43				5.49		6.37			
Run No.				10				12				14				16									
0 - 3 h		372		321		-14		234		259		+11		122		96		-21		236		196		-17	
3 - 22 h		96		118				96		84				17		21				28		13			
22 - 24 h		2002		2722		+36		860		871		+ 1		1292		1548		+20		2003		2742		+37	
Total		2470		3161		+28		1190		1214		+ 2		1431		1655		+16		2267		2951		+30	
Nitrogen (mg)		8.61		8.87				6.39		5.69				3.90		4.48				6.56		7.18			

Table VII. (cont'd)

		C ₂ H ₄ (m μ l)			
		Oxythiamine (1 x 10 ⁻³ M)		Pyridoxal Phosphate (5 x 10 ⁻⁴ M)	
Run No.		17		19	
Period		A	B	A	B
0 - 3 h		296	304	169	212
3 - 22 h		N	N	88	39
22 - 24 h		513	662	830	772
Total		809	966	1087	1023
Nitrogen (mg)		6.23	7.12	5.60	6.30
Run No.		18		20	
0 - 3 h		309	489	268	349
3 - 22 h		75	N	141	N
22 - 24 h		1457	1507	626	585
Total		1841	1996	1035	934
Nitrogen (mg)		5.49	6.01	5.03	4.50

Basic Reaction Mixture

0.5M sucrose

0.125M KH₂PO₄

pH 7.2

0.05M β -alanine

0.002M TPP

0.001M MgSO₄

1.9 x 10⁻³M ATP were added to each flask after initial sonication
Particles were sonicated for 4 min. at 1.2 amps at the beginning of the 0-3 hr. and 22-24 hr. collection periods, respectively

A = control containing particles suspended in basic reaction mixture but without added constituent

B = flask containing the same as A but with added constituent

% = percent increase or decrease in ethylene production

T = trace amount

N = none detectable

10. Effects of Cofactors and Substrates with β -Alanine Decarboxylation System II on Ethylene Production

The second decarboxylation study contained the following basic factors: 0.05 M β -alanine, 2×10^{-3} M TPP, 1×10^{-3} M Mg^{++} and 1×10^{-3} M α -lipoic acid. Factors were then added to this system in the particulate fraction and their effects observed.

Continued evaluation of such cofactor combinations should eventually lead to a combination resulting in maximum stimulation of β -alanine metabolism to ethylene. Although more extensive cofactor combinations could not be studied, the indication derived from the three studies conducted with β -alanine was that careful manipulation of cofactors is required before pronounced stimulation of ethylene production can be realized.

Several complications arise where extensive additions of cofactors are made. There is, for example, the possibility of stimulation of other enzyme reactions. These may be detrimental to ethylene production because intermediates on the ethylene pathway may be withdrawn and also, energy as ATP may be utilized, thus jeopardizing the supply needed for ethylene biosynthesis. There is also the likelihood of increased cofactor antagonism and therefore, it may become necessary to control the amounts of cofactors more critically.

An experimental evaluation of these complications was not attempted because of a lack of time. However, wherever they were thought to be pertinent to the interpretation of results, mention was made to them. Despite the possible shortcomings of this study, valuable information, at least, in the author's estimation was gained about the ethylene producing system.

Results and Discussion

The results of the effects of cofactors with decarboxylation system II are presented in Table VIII (page 146). Briefly, the procedure was to add 0.05M β -alanine, 2×10^{-3} M TPP, 1×10^{-3} M Mg^{++} and 1×10^{-3} M α -lipoic acid to both flasks. Flask B (see Table VIII for designation) also contained the additional factor being evaluated. Then to each flask was pipetted 25 ml of the sonicated tomato particulate suspension.

a) Coenzyme A (7×10^{-5} M) : Runs 1, 2

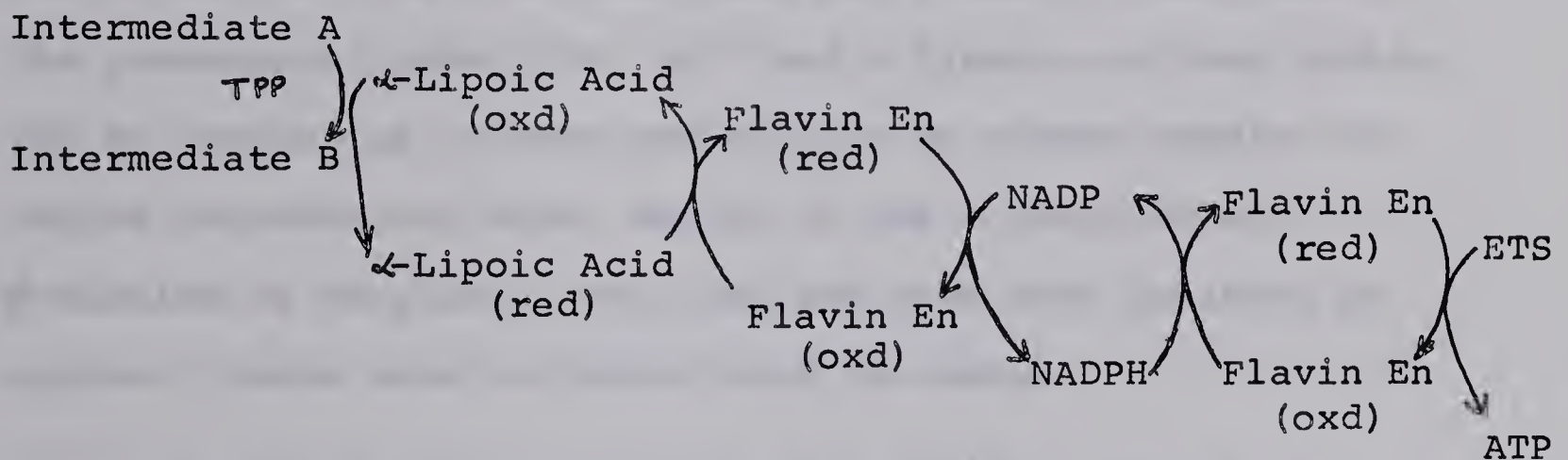
Ethylene production decreased in the presence of coenzyme A.

The effect of CoA remains unchanged from that of previous studies and no further statement will be made. The reader therefore, is referred to Chapters 4, 8 and 9 for the comments made concerning the behavior of the cofactor.

b) Flavin Adenine Dinucleotide ($1.2 \times 10^{-4} \text{M}$) : Runs 3, 4

A slight elevation in the total yield of ethylene for the 22 - 24 hr. period was observed when FAD was present in the particulate fraction, although the initial yield decreased.

The current result agrees with that obtained from the effect of FAD on β -alanine but is opposite to that observed with FAD on β -alanine decarboxylation system I. In the latter study, a significant decrease in ethylene evolution occurred. A probable reaction scheme may facilitate the explanation of the results.



oxd : oxidized form

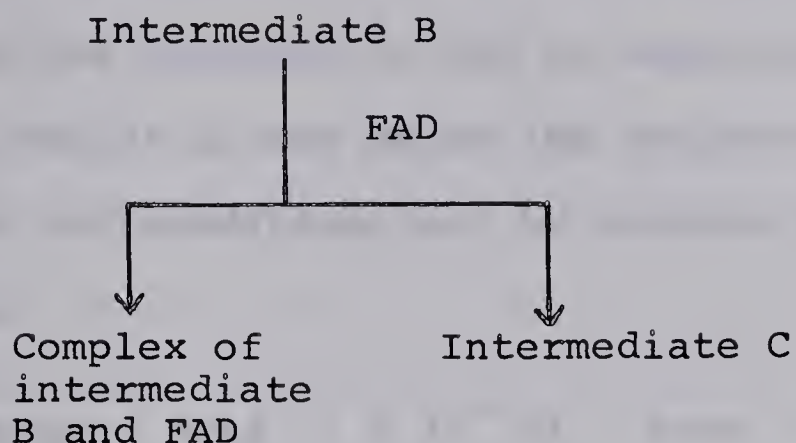
red : reduced form

En : enzyme

ETS : electron transfer system

In system I, which lacks added α -lipoic acid, FAD may have complexed with intermediate A and not allowed it to undergo further oxidation, or it may have induced the formation of another

product from the intermediate. To illustrate the point,



Still another possibility is the formation of a complex with substrate, TPP, Mg^{++} and FAD that may remain undissociated. The presence of added TPP, Mg^{++} and α -lipoic acid may enable FAD to assume its correct position in an enzyme complex or enzyme sequence and thus, assist in the conversion of β -alanine to ethylene. This may not have been achieved in system I where added α -lipoic acid is absent.

c) Flavin Mononucleotide ($2 \times 10^{-4}M$) : Runs 5, 6

A small increase in total production of ethylene was noticed with FMN. However, overnight production (3 - 22 hr) was significantly higher than that of the control.

In all three studies involving β -alanine and FMN stimulation of ethylene evolution has been noticed. Such evidence would seem to imply that an FMN containing enzyme is involved in either the reduction or dehydrogenation of an intermediate leading to ethylene. That an enzyme complex may be involved

in ethylene biosynthesis is again suggested by the pronounced overnight production of the volatile in this experiment. What may be occurring in the presence of FMN is reconstitution of the enzyme complex but at a rate below the optimum value because other factors and conditions may be lacking or require more rigid control.

d) α -Ketoglutaric Acid ($1 \times 10^{-3}M$) : Runs 7, 8

Elevation of ethylene evolution occurred with $10^{-3}M$ α -ketoglutaric acid.

The weak stimulation with α -ketoglutaric acid has been observed several times and probably reflects the need for transamination in ethylene biosynthesis. However, its weak stimulatory effect may be due to a slow rate of transamination of β -alanine, the presence of an endogenous amino group acceptor or a high degree of specificity by the transaminase for the amino group acceptor.

c) Nicotinamide Adenine Dinucleotide ($3 \times 10^{-4}M$) : Runs 9,10

Ethylene production for the 22 -24 hr period was sharply reduced by NAD at $3 \times 10^{-4}M$.

In the previous studies (see Chapter 4, 8 and 9), NAD was usually found to be without effect but in this instance, a severe inhibition of ethylene production was observed. Evidently a cofactor arrangement had been reached that was detrimental to ethylene biogenesis. The key to unlocking

this problem must lie with α -lipoic acid. One possibility is that β -alanine is shunted off into a pathway not leading to ethylene. A second explanation is that a necessary redox chain is impaired by addition of NAD. The third suggestion is that exogenous NAD may be causing a reversal of the electron transport system but why this would occur in the presence of α -lipoic acid and not when the latter is absent is unknown.

f) Nicotinamide Adenine Dinucleotide Phosphate
($4 \times 10^{-4}M$) : Runs 11, 12

The total yield of the olefin was reduced by addition of NADP and a decrease of about 25% occurred during the 22 - 24 hr. period.

The inhibitory effect of NADP contrasts sharply with the stimulatory effects observed in previous studies. Like that of NAD, the inhibition seems to depend upon the presence of α -lipoic acid because in the presence of TPP, Mg^{++} and β -alanine, NADP gave small increases in ethylene production. The reasons cited for the behavior of NAD are also applicable to NADP. Briefly, the probable causes for the inhibitory effect may be one of disruption of a redox chain, reversal of oxidative phosphorylation, production of an intermediate not conducive to ethylene synthesis and promotion of oxidative decarboxylation, a process implicated as detrimental to ethylene biogenesis.

g) Oxalacetic Acid ($1 \times 10^{-3}M$) : Runs 13, 14

Oxalacetic acid at $10^{-3}M$ was inconsistent in its effect on ethylene production. Little to significant stimulation was observed with the organic acid. Whether α -lipoic acid was, in any way, responsible for these effects is unknown but in the absence of the cofactor oxalacetic acid stimulated ethylene production. Valid also is a greater variance in the endogenous supplies of the dicarboxylic acid.

h) Oxythiamine ($1 \times 10^{-3}M$) : Runs 15, 16

The increase in total ethylene production initiated by oxythiamine at $10^{-3}M$ was of the order of 10%.

The effect of oxythiamine remains the same in each of the studies.

i) Pyridoxal Phosphate ($5 \times 10^{-4}M$) : Runs 17, 18

No pronounced effect on ethylene evolution was observed when pyridoxal phosphate was present in the particulate suspension.

Most of the studies employing pyridoxal phosphate disclosed little effect of this compound on ethylene production. Transamination proposed in Chapter 3 must depend on a transaminase that binds pyridoxal phosphate tightly and thus requires no additional source of the cofactor.

Several highlights are evident from the effects of cofactors with β -alanine decarboxylation system II on ethylene

biogenesis. FAD, which was inhibitory in system I, gave slight stimulation in system II. The stimulatory role of FMN remained unchanged. Both NAD and NADP were significantly inhibitory in system II, as compared to no effect and slight augmentation, respectively, in system I. Apparently, the addition of NAD or NADP and α -lipoic acid results in an event deleterious to ethylene biosynthesis. The inhibitory effect of CoA was unchanged and a lack of effect with pyridoxal phosphate was observed again. The two amino group acceptors, oxalacetic and α -ketoglutaric acid, elevated synthesis of ethylene weakly, a result also obtained with system I.

Table VIII. Effects of Cofactors and Substrates with β -Alanine Decarboxylation System II on Ethylene Production by a Particulate Fraction from Tomatoes

C ₂ H ₄ (mul)													
		Coenzyme A (7 x 10 ⁻⁵ M)			Flavin Adenine Dinucleotide (1.2 x 10 ⁻⁴ M)			Flavin Mononucleotide (2 x 10 ⁻⁴ M)			α-Ketoglutaric Acid (1 x 10 ⁻³ M)		
Run No.		1		3		5		7					
Period	A	B	%	A	B	%	A	B	%	A	B	%	
0 - 3 h	371	300	-19	399	298	-25	167	191	+14	299	293	- 2	
3 - 22 h	42	23		N	N		62	281		12	9		
22 - 24 h	667	575	-14	1878	1980	+ 5	1890	2159	+14	540	663	+23	
Total	1080	898	-17	2277	2288		2119	2631	+24	851	995	+17	
Nitrogen (mg)	7.01	7.10		7.18	7.08		5.25	5.29		6.23	5.39		
Run No.													
		2		4		6		8					
0 - 3 h	276	261	- 5	167	146	-13	171	180	+ 5	220	219		
3 - 22 h	20	25		62	107		90	167		N	N		
22 - 24 h	1004	800	-20	1890	2122	+12	1950	2075	+ 6	665	930	+40	
Total	1300	1096	-16	2119	2385	+13	2211	2422	+10	885	1149	+30	
Nitrogen (mg)	7.98	7.94		5.25	5.11		4.20	5.34		5.09	4.62		

Table VIII. (cont'd)

C ₂ H ₄ (mμl)												
Nicotinamide Adenine Dinucleotide (3 x 10 ⁻⁴ M)				Nicotinamide Adenine Dinucleotide Phosphate (4 x 10 ⁻⁴ M)				Oxalacetic Acid (1 x 10 ⁻³ M)			Oxythiamine (1 x 10 ⁻³ M)	
Run No.	9			11			13			15		
Period	A	B	%	A	B	%	A	B	%	A	B	%
0 - 3 h	321	366	+14	321	321		276	270	- 4	276	253	- 8
3 - 22 h	118	96		118	90		N	N		N	118	
22 - 24 h	2722	2137	-21	2722	1980	-27	1597	1600		1597	1724	+ 8
Total	3161	2599	-18	3161	2491	-21	1875	1870		1875	2095	+12
Nitrogen (mg)	8.87	8.49		8.87	9.01		4.35	4.09		4.35	4.96	
Run No.	10			12			14			16		
0 - 3 h	171	129	-25	197	205	+ 4	220	225	+ 2	231	160	-31
3 - 22 h	90	51		124	90		N	39		73	126	
22 - 24 h	1950	1474	-24	3157	2401	-24	665	825	+24	815	840	+ 3
Total	2211	1654	-25	3578	2696	-25	885	1089	+23	1019	1126	+11
Nitrogen (mg)	4.20	3.64		4.76	5.81		5.09	4.56		3.48	3.85	

Table VIII. (cont'd)

C ₂ H ₄ (m μ l)			
Pyridoxal Phosphate (5 x 10 ⁻⁴ M)			
Run No.	17		
Period	A	B	%
0 - 3 h	371	347	- 6
3 - 22 h	42	35	
22 - 24 h	667	625	- 6
Total	1080	1007	- 7
Nitrogen (mg)	7.01	7.28	
Run No.	18		
0 - 3 h	276	276	
3 - 22 h	20	30	
22 - 24 h	1004	892	-11
Total	1300	1198	- 8
Nitrogen (mg)	7.98	7.63	

Basic Reaction Mixture

0.05M sucrose

0.125M KH₂PO₄

pH 7.2

1.9 x 10⁻³M ATP

0.05M p-alanine

2 x 10⁻³M TPP

1 x 10⁻³M MgSO₄

1 x 10⁻³M α -lipoic acid

were also added to each flask after initial sonication Particles were sonicated for 4 min. at 1.2 amp at the beginning of the 0-3 hr. and 22-24 hr. collection periods, respectively.

A = control containing particles suspended in basic reaction mixture but without added constituent

B = flask containing the same as A but with added constituent

% = percent increase or decrease in ethylene production

T = trace amount

N = none detectable

11. The Effects of Ageing on Ethylene Production in the Presence of Some Added Factors

Several experiments were conducted in which the tomato particles were not sonicated initially but allowed to age for 22 hours before being subjected to a sonic treatment. Production of ethylene during the ageing period by the initially intact particles was compared to that obtained with particles initially sonicated. Furthermore, the evolution of the volatile during the 22 - 24 hr. period was compared to that of particles initially disrupted by sonication and then resonicated after the ageing period. Disintegration to some extent would occur with the initially intact particles and one might, therefore, obtain some indication as to how limited disintegration of the particles affects ethylene production. Partial disintegration would probably result from the swelling of the particles, a phenomenon studied by several workers (197, 198, 199, 200, 201, 202). An increase in NADH level (203) and in ATPase activity (183) have been reported for aged mitochondria. In fact, many biochemical events are dependent upon the integrity of the mitochondrion (204). Consequently, the results may disclose whether a degree of particle organization is essential for sustained production of the volatile or, at least, for the accumulation of a precursor that may be rapidly converted to ethylene upon complete disruption of the particles by sonication. Sonication of particles is known to disrupt activity of the TCA cycle (128) but oxidative phosphorylation still continues (128, 172, 173). Whether ethylene

production and particle integrity are related may become evident from the results.

Results and Discussions

The results obtained on the effects of ageing of some factors on ethylene production by the tomato fraction are assembled in Table IX (page 154).

a) Adenosine Triphosphate (sodium salt) (1.9×10^{-3} M) :

Runs 1, 2.

ATP at 1.9×10^{-3} M elevated ethylene production slightly for the 3 - 22 hr. period and substantially for the 22 - 24 hr. period. Total ethylene production increased by 70% or more in the presence of the cofactor.

Chandra, Spencer, and Meheriuk (155) previously reported ATP to enhance ethylene production by the particulate fraction from tomatoes. The cofactor was added to an initially sonicated suspension and ethylene collected for 20 hours. The stimulatory effect of ATP was particularly evident during the 2 - 20 hr. collection period. Since the mode of the two sets of experiments differ no direct comparison can be made. Nevertheless, the significance of ATP to ethylene production is corroborated by the results from both sets of experiments.

b) Thiamine Pyrophosphate (2×10^{-3} M), Mg^{++} (as $MgSO_4$)

(1×10^{-3} M) : Runs 3, 4.

Total production of ethylene was elevated by the addition of 2×10^{-3} M TPP and 1×10^{-3} M Mg^{++} .

The increase in the yield of ethylene effected by TPP and Mg^{++} was approximately the same magnitude as that observed with particles that had been sonicated twice (see Chp. 4). Perhaps a larger stimulatory effect was not realized for several reasons. Thiamine pyrophosphate may have been unable to readily penetrate into the particles, however, information on this aspect is lacking. There may also have been an ample endogenous supply of the cofactor. Moreover, a limited amount of an intermediate leading to ethylene may have restricted the stimulatory effect possible. Finally, a more organized system than that present with initially sonicated particles may have no influence on the stimulatory effect of TPP and Mg^{++} .

c) β -Alanine (0.05M), TPP (2×10^{-3} M), Mg^{++} (1×10^{-3} M) :
Runs 5, 6.

A pronounced increase in ethylene production was observed when β -alanine, TPP and Mg^{++} were added to the particulate fraction.

The result certainly indicates the marked influence of an added substrate in the presence of TPP and Mg^{++} on the ethylene production by initially intact particles. The increase exceeds by a considerable degree that observed with particles containing the same factors that were sonicated twice. It would seem that lack of a precursor may have been the cause for the low stimulatory effect of TPP and Mg^{++} noted in runs 3 and 4. Moreover, in the presence of the three factors, particles that had been sonicated initially produced less ethylene than those

which were intact initially. One might be tempted to suggest that a higher degree of organization present in the initially intact particles may have resulted in the accumulation of a precursor that was rapidly converted to ethylene upon complete disintegration of the particles by sonication. However, this behavior may only occur with β -alanine as a substrate and additional experiments are required to assess the validity of the proposal.

Although a more extensive study was not completed the few results obtained are, nonetheless, of considerable importance. First, the requirement of energy (as ATP) in the biosynthesis of ethylene was confirmed. Although it would be difficult to know, ATP may have exerted part of its stimulatory effect by virtue of its ability to prevent the swelling of the particles and would, thus, reduce the extent of disintegration within the suspension. Reversal of mitochondrial swelling by ATP has been reported by several people (197, 198, 201, 202). Secondly, the need for an organized enzyme system for enhanced metabolism of β -alanine (with TPP and Mg^{++} present) to ethylene was suggested. Moreover, it would appear that ethylene production is dependent upon the accumulation of a precursor that is rapidly converted to ethylene upon sonication of the particles. This is somewhat analogous to in vivo conditions where disintegration of mitochondria occur during the climacteric period (96, 193), a period of highest ethylene production.

Why resonication of a suspension disrupted initially should result in a large outburst of ethylene is unknown. Electron micrographs have revealed complete disruption of tomato particles after initial sonication and further disintegration of the disrupted particles may appear as one cause for the large outburst of ethylene. Related to this latter aspect would be the liberation of any ethylene bound to the disrupted particles. Perhaps an enzyme complex formed (this has been suggested in Chp. 8, 9, and 10) is activated but the biophysical basis of stimulation is open to conjecture. Sonication, nevertheless, still remains as an effective means of disintegrating tomato particulate fractions which for reasons unknown are very active in the production of ethylene (65, 66, 97).

Table IX. The Effect of Ageing in the Presence of Several Constituents on Ethylene Production by a Particulate Fraction from Tomatoes

C ₂ H ₄ (μl)									
Run No.		1		3		5			
Period		Adenosine Tri-phosphate* (1.9 x 10 ⁻³ M)		Thiamine Pyrophosphate (2 x 10 ⁻³ M) Mg ⁺⁺ (1 x 10 ⁻³ M)		β-alanine (0.05 M) TPP (2 x 10 ⁻³ M) Mg ⁺⁺ (1 x 10 ⁻³ M)			
		A	B	%	A	B	%	A	B
0 - 3 h		N	N	-	7	7	-	11	9
3 - 22 h		N	23	-	14	7	-	N	25
22-24 h		1034	1699	+64	1628	2102	+29	1510	4463
Total		1034	1722	+67	1649	2116	+28	1521	4497
Nitrogen (mg)		2.83	3.06		8.66	7.94		6.13	6.43
Run No.		2		4		6			
0 - 3 h		N	N	-	23	17	-	N	N
3 - 22 h		17	28	-	34	34	-	23	T
22-24 h		883	1652	+87	744	996	+34	990	2188
Total		900	1680	+86	801	1047	+31	1013	2188
Nitrogen (mg)		6.39	7.53		7.70	6.93		7.09	6.12

Basic Reaction Mixture
0.5 M sucrose
0.125 M KH₂PO₄
pH 7.2
1.9 x 10⁻³ M ATP
was added to each flask after suspension of particles

Particles were sonicated for 4 min and 1.2 amp at the beginning of the 22-24 hr. collection period

T = trace amount

N = none detected

* ATP was not added to A

A = control containing particles suspended in basis reaction mixture but without added factors

B = flask containing the same as A but with added factors

% = percent decrease or increase in ethylene production

12. The Identification, Estimation, and Possible Conversion to Ethylene, of Ethanol Produced by the Tomato Particulate Fraction.

a) Identification:

The mass spectrogram of volatiles produced by the particulate fraction and collected on activated alumina is illustrated in Fig. 1 (page 161). Although only verification of ethanol was sought other volatiles not identified were revealed. The peak at 31 can represent methyl, ethyl, and propyl alcohol as well as the methyl and ethyl esters of ethanol. Ethanol, also, gives a peak at 45, a peak observed on the spectrum. Since many alcohols and their esters may be present it is impossible to identify any of the volatiles on the basis of the peak heights observed on the mass spectrogram. About all one is allowed to say is that ethanol is very likely one of the volatiles detected. Additional means must be utilized before positive identification of any volatile can be achieved. These have been employed by a number of people and several alcohols have been identified as constituents of plant volatiles (205, 206, 207, 208).

b) Estimation:

i) Ethanol Content of the Suspension:

Determination of the ethanol content in the particulate suspension was executed with a two fold purpose. The first was to observe the behavior of the suspension in the production of the alcohol and the second was to enable assessment of its possible contribution to the total yield of ethylene. Values obtained from a preparation divided into

two equal portions are presented in Table X (page 159).

Ethanol content remained fairly constant for three hours after initial sonication but increased appreciably after resonation of the suspension at 22 hr. At the end of the 22-23 hr. period the ethanol content was $1.3 \times 10^{-3}M$ at its highest value. Subsequent measurement revealed a drop in the ethanol concentration during the 23-24 hr. period to a value similar to that observed after sonication at 22 hr. Some of the ethanol may have been converted to ethylene in view of the reports by other workers that it promotes ethylene production in some organisms (40,52,50).

ii) Ethanol Content of Expired Volatiles:

The ethanol contents of the water samples (in which volatiles evolved by the particulate fraction were collected) remained relatively constant (see Table XI) (page 160).

The possible contribution of ethanol collected in mercuric perchlorate to the total yield of ethylene as postulated by Burg and Burg (95) can be calculated in the following manner.

(1) 1 mole ethanol \rightarrow 1 mole ethylene

at 730 mm Hg and 25°C 1 mole of ethylene will occupy a volume of V_1 , which can be calculated as follows:

$$\frac{P_o V_o}{T_o} = \frac{P_1 V_1}{T_1} \quad \text{where } P_o = 760 \text{ mm} \quad P_1 = 730 \text{ mm}$$

$$V_o = 22.4 \text{ L} \quad V_1 = ? \text{ unknown volume of } C_2H_2$$

$$T_o = 273^\circ C \quad T_1 = 298^\circ C$$

$$\text{so that } V_1 = \frac{P_o}{P_1} \cdot \frac{T_1}{T_o} \cdot \frac{V_o}{1}$$

$$= 760/730 \times 298/273 \times 22.4/1$$

$$= 25.4 \ell$$

(2) If an average value of 0.025% wt/vol of ethanol is taken, then in a 4.0 sample of perchlorate reagent one would have

$$\frac{.025}{100} \times \frac{4.0}{1} \text{ or } 0.001 \text{ gms or } \frac{.001}{46} \text{ moles of ethanol}$$

Assuming a maximum conversion of 0.02% (95) for the ethanol to ethylene, one would have,

$$\frac{0.001}{46} \times \frac{0.02}{1} \times \frac{25.4}{1} = 1.1 \times 10^{-5} \text{ L or } 11 \mu\ell \text{ of ethylene produced.}$$

11 $\mu\ell$ would amount to 11,000 $\mu\ell$ of ethylene.

The latter value in terms of the usual amounts of ethylene collected from a particulate suspension is large and, theoretically, could constitute a major portion of the total ethylene. Additional experiments were conducted to determine the actual contribution and are reported next.

c) Dehydration Studies with Ethanol:

In the preceding section it was stated that 0.001 gm of ethanol may be present in the 4.0 ml quantity of mercuric perchlorate reagent. In terms of a 95% solution of ethanol, 0.001 gm of the alcohol amounts to 0.001 ml or $1 \mu\ell$. It was, however, decided to add $10 \mu\ell$ of the alcohol to 4.0 ml of mercuric perchlorate reagent, as a rigorous test. Individual samples aged for 24, 48, 72 and 96 hr. and then liberated with 1 ml LiCl revealed no peaks for ethylene on the gas chromatogram. Since the alcohol contents of these samples were ten fold greater than that detected in the water samples it can be concluded that no contribution to the total yield of ethylene from the dehydration of ethanol in mercuric perchlorate is likely.

TABLE X

The Ethanol Content of a Particulate Fraction from Tomatoes

Ethanol (% wt/vol)		
	Sample A	Sample B
* (1) initial, after sonication	0.004	0.004
(2) end of 0-3 hr.	0.004	0.003
* (3) immediately after resonation at 22 hr.	0.031	0.038
(4) end of 22-23 hr.	0.058	0.059
(5) end of 22-24 hr.	0.036	0.041

Particles were suspended in sucrose phosphate buffer (0.5 M sucrose, 0.5 M KH_2PO_4 , adjusted to pH 7.2 with NaOH); 1.9×10^{-3} M ATP was added to each sample after initial sonication.

A and B are equal portions of a particulate fraction preparation.

* Suspensions were sonicated for 4 minutes at 1.2 amperes initially and at 22 hr.

TABLE XI

Ethanol Content of Volatiles Evolved by a Particulate
Fraction from Tomatoes

Ethanol (% wt/vol)		
Period	A	B
0-3	0.018	0.024
3-22	0.024	0.034
22-23	0.017	0.025
23-24	0.024	0.022

Particles were suspended in
sucrose phosphate buffer com-
posed of

0.5 M sucrose

0.125 M KH_2PO_4

pH 7.2 (with NaOH)

and sonicated for 4 min.

at 1.2 amps. and then

divided into two equal

portions of 25 ml.

Added to each flask was

1.9×10^{-3} M ATP.

Suspensions were resoni-

cated at beginning of 22-23

hr. period.

A and B are equal
portions of a parti-
culate fraction prep-
aration. Volatiles
were collected in de-
ionized water.

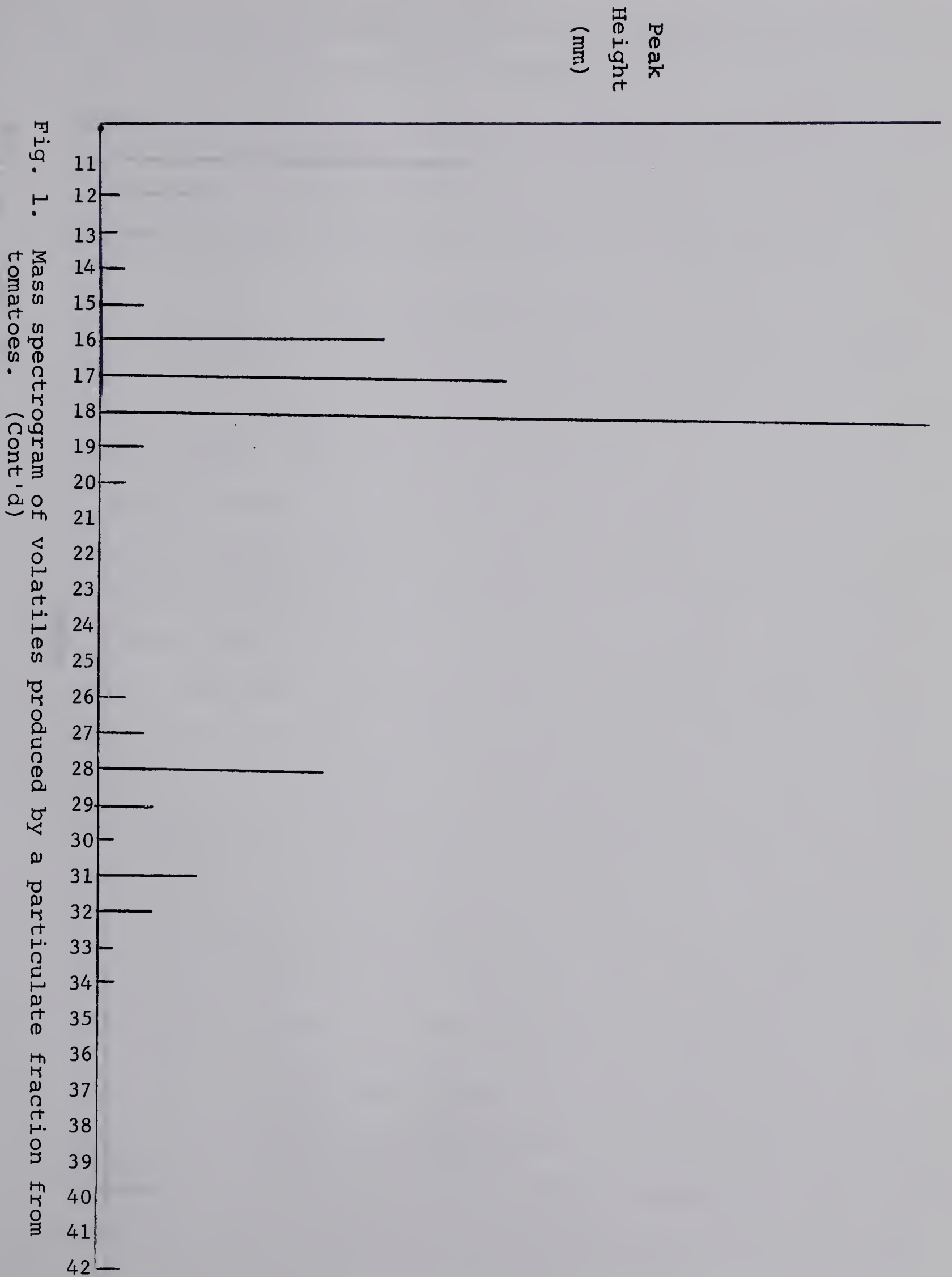
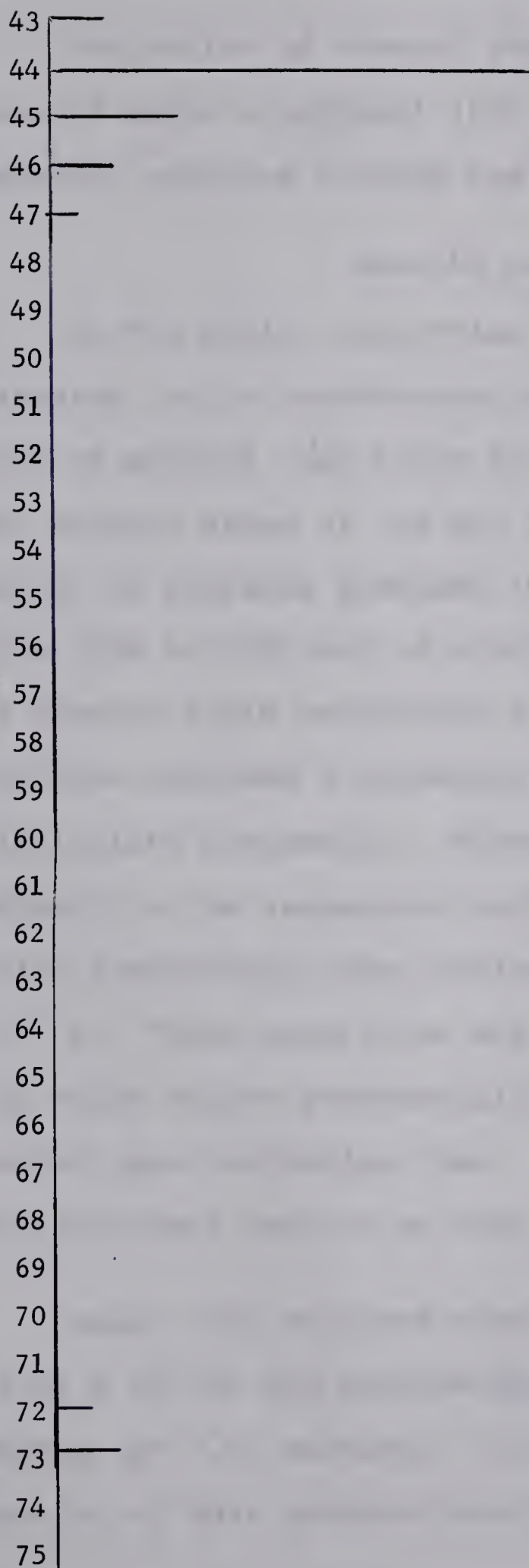


Fig. 1. (Cont'd)

Mass



13. The Effect of Sonication of Several Cofactors and Substrates on Ethylene Production.

Sonication of several cofactors and substrates was made in view of Meigh's proposal (69) that sonication, itself, could generate ethylene through the formation of free radicals.

Results and Discussion

On the whole, sonication of the cofactors and substrates revealed low to non-detected amounts of ethylene with the exception of ethanol (see Table XII (page 164)). One may ask how the average value of 300 μ l for ethanol compares with the total amount of ethylene produced in a 24 hr. period. Usually, values from 1200 to 2000 μ l of ethylene were obtained and sonication of ethanol could contribute significantly to the total yield of ethylene provided a concentration of 0.05 M was present in the particulate suspension. However, the highest value detected for ethanol in the suspension was 0.059% or 1.3×10^{-3} and the highest value immediately after sonication at 22 hr. was 0.039% or 8.4×10^{-4} M. These quantities are considerably lower than 0.05 M and one would expect substantially lower quantities of ethylene generated upon sonicating them. In fact, the actual yields may be insignificant insofar as total ethylene production is concerned.

Meigh (69) detected ethylene upon sonication of an ATP (1.98×10^{-3} M) and sucrose-phosphate (0.5 M sucrose, 0.125 M KH_2PO_4 , pH 7.0) mixture. In the authors experimental work sonication of this mixture resulted in non-detectable to minimum

detectable amounts of ethylene and thus indicates the inconsistency of sonication. However, Meigh's gas chromatograph is considerably more sensitive than the one used by the author and it is likely that detectable amounts of ethylene for him would be undetectable to the author. Moreover, Chandra et al (155) were critical of his method as it did not eliminate the ethylene contamination from the air, a feature eliminated in the procedure followed by Chandra et al. Furthermore, the type of sonic oscillator used, the period of sonication and the volume sonicated would all have a marked influence upon the generation of ethylene.

A preliminary experiment with the tomato fraction disclosed that TCA (trichloroacetic acid) added after initial sonication (final concentration of TCA was 14%) did not alter initial production of ethylene but almost completely inhibited production for the 22 - 24 hr. period. Since inactivation of the enzymes would be extant, it is evident that sonication of the denatured protein is not a contributing factor to ethylene production.

Table XII. Ethylene Production by Sonication of Several Constituents

Run No.	β -alanine (0.05 M)	Aspartic Acid (0.05 M)	Ethanol (0.05 M)	C ₂ H ₄ μ l				ATP (1.9 x 10 ⁻³ M)	TPP (2 x 10 ⁻³ M) Mg (1 x 10 ⁻³ M)
				Galactose (0.05 M)	Glutamic Acid (0.05 M)	Propionic Acid (0.05 M)			
1	11	28	288	20	51	69	N	N	
2	N	N	350	68	T	53	2	N	
3	56	21	309	40	38	53	N	18	
4	-	9	-	-	N	-	-	N	
5	-	39	-	-	N	-	-	14	

Run No.	C ₂ H ₄ μ l			
	Pyridoxal Phosphate (5 x 10 ⁻⁴ M)	NADP (4.0 x 10 ⁻⁴ M)	α -lipoic acid (1 x 10 ⁻³ M)	
1	11	68	56	
2	12	N	62	
3	5	51	-	
4	-	-	-	
5	-	-	-	

Constituents were dissolved in 25 ml of buffer (0.5 M sucrose, 0.125 M KH₂PO₄, pH 7.2), aged for 22 hours, then sonicated for 4 min. at 1.2 amp. and ethylene collected for 2 hr.

T = Trace amount.

N = None detected.

14. Confirmation of the Identity of Ethylene.

In addition to gas chromatography, two methods used in the identification of the volatile as ethylene were testing of the absorption in bromine water and mass spectrometry.

a) Absorption in Bromine Water.

Studies with an ethylene standard and the unknown sample revealed gas chromatographic peaks at identical retention times. However, gas samples from both when injected into individual samples and shaken and then withdrawn to be analyzed by gas chromatography gave no peaks on the chromatogram. Complete absorption was achieved in less than 30 seconds. The observation of peaks on the chromatogram at a specific retention time for samples withdrawn from a standard and an unknown, and the disappearance of these peaks when gas samples were shaken in bromine water was taken as confirmation that the volatile collected was an olefin.

b) Mass Spectrometric Analysis.

The mass spectrogram of a standard containing 780 μ l of ethylene is presented in Figure 2. Peaks that represent ethylene are 26 and 27 and they should be approximately in a 1:1 ratio to each other. The mass spectrogram of an unknown sample is presented in Figure 3. Peaks at 26 and 27 are evident for the unknown sample and thus confirm the identity of the volatile as ethylene. Both samples were prepared by the "break-seal" method described on Page 25. The isopentane-liquid air method could not be completed because of mechanical failure of the mass spectrometer.

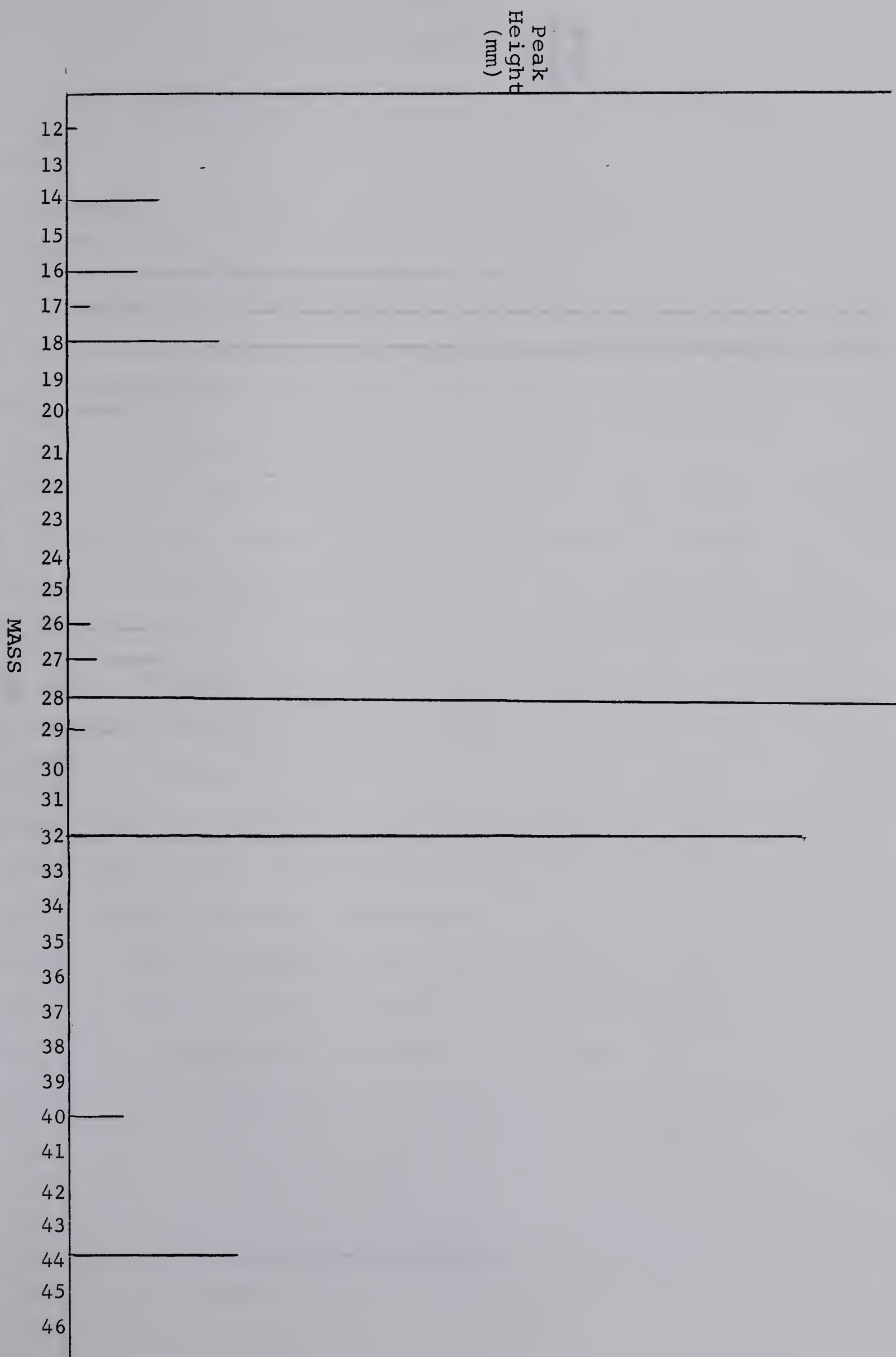
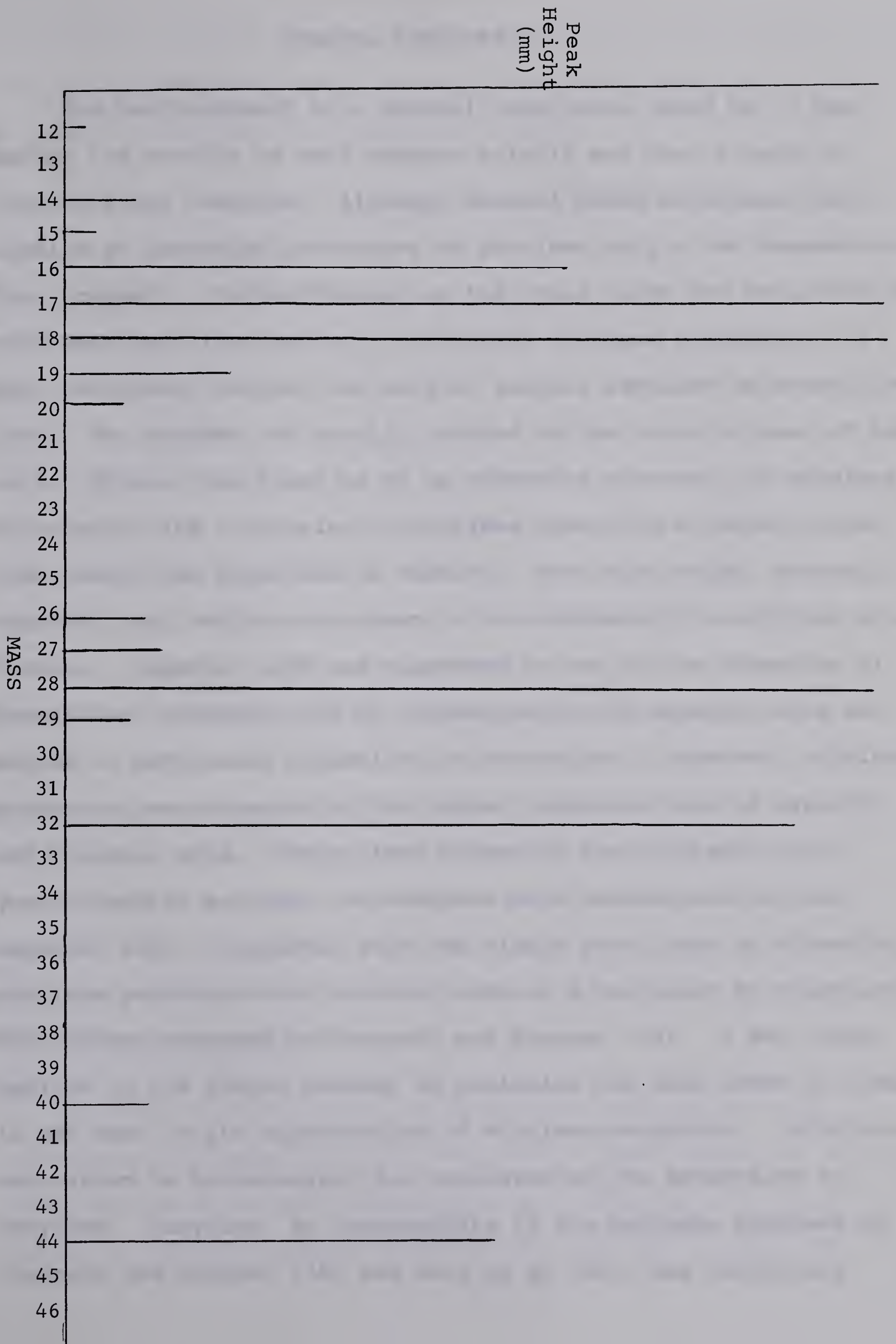


Figure 2. Mass spectrogram of standard sample of Ethylene.

Figure 3. Mass spectrogram of unknown sample of collected volatile.



General Conclusion

The best approach to a general conclusion would be to summarize the results of each chapter briefly and then attempt to correlate any features. Although several added substrates were studied as potential precursors of ethylene only a few demonstrated this property. Carbohydrates, on the whole, with the exception of galactose were ineffective in promoting ethylene production and may, therefore, reflect the lack of enzymes required to metabolize them. The enzymes are usually located in the soluble phase of the cell. Ethanol was found to be an effective precursor of ethylene but whether its conversion to ethylene occurs by a pathway other than acetyl-CoA formation is unknown. The amino acids, glutamic, aspartic, and serine were shown to be stimulatory to ethylene production. Aspartic acid was suggested to act in the formation of homoserine, glutamic acid by transamination to aspartic acid and serine in methionine metabolism to homoserine. Moreover, ethylene production was enhanced by the higher concentrations of aspartic and glutamic acid. Though less effective than glutamic acid, γ -aminobutyric acid may transaminate with oxalacetate to form aspartic acid. Propionic acid was highly proficient in elevating ethylene production and probably acts as a precursor by entry into the pathway proposed by Thompson and Spencer (74). A key intermediate in the latter pathway is β -alanine but when added by itself it was weak in its augmentation of ethylene evolution. Cofactors were shown to be necessary for accelerating its metabolism to ethylene. Acrylate, an intermediate in the pathways proposed by Thompson and Spencer (74) and Wang et al (54), was inhibitory

to ethylene production but a high reactivity of the compound coupled with a level that was deleterious to enzyme activity was cited as a probable cause for the inhibitory effect.

A number of cofactors were studied and it was found that the highest stimulatory effect on ethylene production was induced on addition of TPP and Mg^{++} together. Slight elevations in the yield of the olefin from NADP and α -lipoic acid were also observed. Stimulatory effects by TPP and Mg^{++} and by α -lipoic acid usually are an indication of decarboxylase activity, but it was not possible to determine whether it was of the oxidative or non-oxidative type in our reaction system. An NADP-linked dehydrogenase is very likely involved in the biosynthesis of ethylene. A significant overnight production of ethylene in the presence of FMN suggested that reconstitution of either an enzyme complex or enzyme sequence may have been inaugurated in the presence of the prosthetic group. Moreover, support for such a reconstitution was obtained from the synergistic effect observed between CoA and NAD or NADP. Severe inhibition by reduced glutathione implied a need for some unreduced disulfide linkages in enzymes associated in ethylene biogenesis. The mild inhibitory effect of CoA was explained on the basis of an acyl derivative of an intermediate that interferes with ethylene biosynthesis.

Considerable work with inhibitors was done and several interesting features emerged. The role of thiol groups in ethylene synthesis was amply demonstrated by the inhibitory effects of arsenite, iodoacetamide, p-CMB, mercuric ions and silver ions.

The importance of carbonyl groups was disclosed by the inhibition of ethylene production with hydroxylamine, semicarbazide and cyanide. Carbonyl groups could involve proteins, cofactors and substrates. Chelation of a cation or metalloprotein was suggested by the actions of azide, cyanide, fluoride and DIECA. Transaminase activity was revealed by cycloserine and aminooxyacetic acid. Inhibition of the cytochrome system is a distinct possibility in view of the inhibitory effects of azide and cyanide. Lack of substantial inhibition with monofluoroacetate and malonic acid led to the proposal that a considerable portion of the TCA cycle is not essential to ethylene biosynthesis. A need for disulfide linkages was also indicated by cyanide.

No cations were found to stimulate ethylene production and in fact, most were inhibitory, probably because of their binding of the thiol groups on enzymes. It is likely that sufficient endogenous supplies of the necessary cations are present in the preparations. However, Mg^{++} was found to be stimulatory in the presence of certain levels of TPP.

A rather broad pH range was noticed for ethylene evolution and values from pH 7.0 to 5.0 were found to sustain production. However, at pH 3.0 ethylene production was severely inhibited. Alkaline pH values were highly deleterious to the production of the volatile and one might suspect that inactive states of enzymes, unavailability of substrates, and denaturation of cofactors were responsible for the pronounced decrease in ethylene production at the alkaline pH values.

Three studies with β -alanine were completed in an attempt to reconstruct an enzyme sequence or enzyme complex that would result in maximum metabolism of β -alanine to ethylene. In the first study various factors were added with β -alanine to the particulate fraction. A significant stimulation in the evolution of the olefin was observed when TPP and Mg^{++} were added as factors. Weak stimulatory effects were also noticed with pyridoxal phosphate, oxalacetic acid, α -ketoglutaric acid, α -lipoic acid, NADP, FAD, and FMN. CoA was found to be inhibitory to ethylene production.

The second study involved the addition of some factors with β -alanine, TPP and Mg^{++} to the tomato fraction. Weak stimulatory effects were obtained with α -lipoic acid, NADP, FMN and α -ketoglutaric acid. Oxalacetic acid, on the other hand, gave an appreciable increase in ethylene production. The effect of CoA remained unchanged but that of FAD now became inhibitory. In addition, pyridoxal phosphate was not stimulatory.

The third study involved the addition of some factors with β -alanine, TPP, Mg^{++} and α -lipoic acid to the particulate fraction. Weak stimulatory effects were observed with FAD, FMN, oxalacetic acid and α -ketoglutaric acid. However, addition of NADP resulted in severe inhibition of ethylene production. The effects of pyridoxal phosphate and CoA repeated themselves.

Several pertinent features became obvious from the three studies. First, FAD in the presence of TPP and Mg^{++} caused a decrease in ethylene production, but not when α -lipoic acid was present. Second, NADP, found to be stimulatory in the presence

of TPP and Mg^{++} , was appreciably inhibitory when α -lipoic acid was also added as a factor. Direct or indirect antagonistic effects would appear to exist between NADP and α -lipoic acid and between FAD and TPP and Mg^{++} . More research is needed to determine the cause of this behavior. Evidently, reconstitution of a holoenzyme or an enzyme complex is an intricate procedure and requires careful manipulation of the various factors. Successful reconstitutions were apparently achieved on the addition of TPP, Mg^{++} , α -lipoic acid and FAD or FMN, and also with TPP, Mg^{++} and NADP. The inhibitory effect of CoA was explained in terms of promotion of oxidative decarboxylation, a process that may be detrimental to ethylene production or of mediation of an event that interferes with the production. Inconsistent effects with pyridoxal phosphate were taken as a reflection of variations in the endogenous contents of the prosthetic group.

A limited study was done in which the particulate fraction containing added factors was allowed to age for 22 hours before sonication. The main purpose behind this study was to determine whether ethylene production and the integrity of the particles were related phenomena. A significant stimulation in ethylene production was observed with ATP. Maintenance of particle integrity and a supply of energy needed for ethylene biosynthesis were proposed as causes of the stimulation. The effect of TPP and Mg^{++} was the same as that obtained with initially sonicated particles. A very pronounced stimulatory effect was observed with β -alanine, TPP and Mg^{++} . The result was regarded as an indication that a higher degree of organization (present in the initially intact

but not the initially sonicated particles) resulted in the accumulation of a precursor that was rapidly converted to ethylene upon complete disintegration of the particles. The assumption is not unreasonable since considerably less stimulation was observed with an initially disrupted suspension containing these factors.

An evaluation of the possible contribution to the total ethylene production from the dehydration of ethanol in mercuric perchlorate was made. Although the theoretical yield possible from the ethanol content of the volatiles evolved by the suspension was significant, experiments revealed that any contribution to the total ethylene production from the dehydration of ethanol was not evident on the basis of conversion reported by Burg and Burg (95).

The effects of sonication of several cofactors and substrates individually revealed that little or no ethylene was produced. However, 0.05 M ethanol when sonicated did give a significant quantity of ethylene, but since the actual ethanol content of the particulate suspension was considerably less than 0.05 M it was assumed that the contribution of the sonication of ethanol to the total yield of ethylene was minor.

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